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The influence of regulatory T cells on remyelination in the central nervous system

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Contents

Abstract

The aim of this project is to study whether Regulatory T cells (Treg) influence remyelination following a demyelinating insult of the central nervous system (CNS). Recent studies have shown that biopsies of demyelinated lesions taken from Multiple Sclerosis (MS) patients contain Treg [1]. They can also be observed in sites of inflammation within the CNS of mice with Experimental Autoimmune Encephalomyelitis (EAE), a model of MS in which CNS inflammation is induced in animals. Since these cells are found in sites of demyelination and secrete a number of cytokines known to affect oligodendrocyte precursor cell (OPC) and oligodendrocyte cell function (Table 1), it is important to understand if and how they influence repair mechanisms such as remyelination.

I have studied the effect that cytokines secreted by Treg have on OPC biology. I have approached this two ways: first by adding conditioned media from in vitro primary cultures of Treg to in vitro primary cultures of OPCs, to study the effects of a full complement of Treg-secreted cytokines on OPCs, and second by choosing the candidate molecule Leukaemia inhibitory factor (LIF), delivered directly to OPCs using nanoparticles. Studies in the past have similarly analysed the effects of single cytokines on OPC behaviour and morphology [2,3] and conditioned media from other T cell types have been added to microglia [4].

In the future, this work will be extended to studying the effect of Treg and LIF nanoparticles in mouse brain slices ex vivo and in an in vivo mouse model of demyelination.

Chapter 1: Background

1.1 Myelin

1.1.1 Structure of Myelin

Axons larger than about 1μm in diameter in the mammalian nervous system are wrapped in a fatty membrane known as myelin. Myelin has several important functions which include the provision of trophic support to axons[5,32] and increasing the rate of axonal conduction. Structurally, myelin is made from multiple layers of plasma membrane, tightly bound to one another by transmembrane proteins such as proteolipid protein (PLP). In the human peripheral nervous system, myelin is produced by Schwann cells that each wrap around a single axon, and in the central nervous system (CNS) by oligodendrocytes (OLs) which each can form multiple myelin sheaths around different axons. Oligodendrocyte cells are post-mitotic, and, under normal conditions, have great longevity. When forming a new myelin sheath, each oligodendrocyte process first contacts an unmyelinated section of an axon. Following contact, the process extends laterally along an axon and finally extends around the axon forming a continuous spiral of plasma membrane filled with myelin proteins. Some of these proteins, such as PLP and myelin basic protein (MBP), maintain the structure of the myelin sheath through cross-linking between layers of membrane (see Fig. 1, adapted from [6,7]). Proteins such as MBP in the intracellular space of the myelin sheath create the 'major dense lines' observed on electron micrograph images of myelin. Genetic ablation of MBP causes a severe and fatal hypomyelination phenotype in mice, the

so-called 'shiverer' mouse [8,9]. On the other hand, mice that lack PLP show no symptoms other

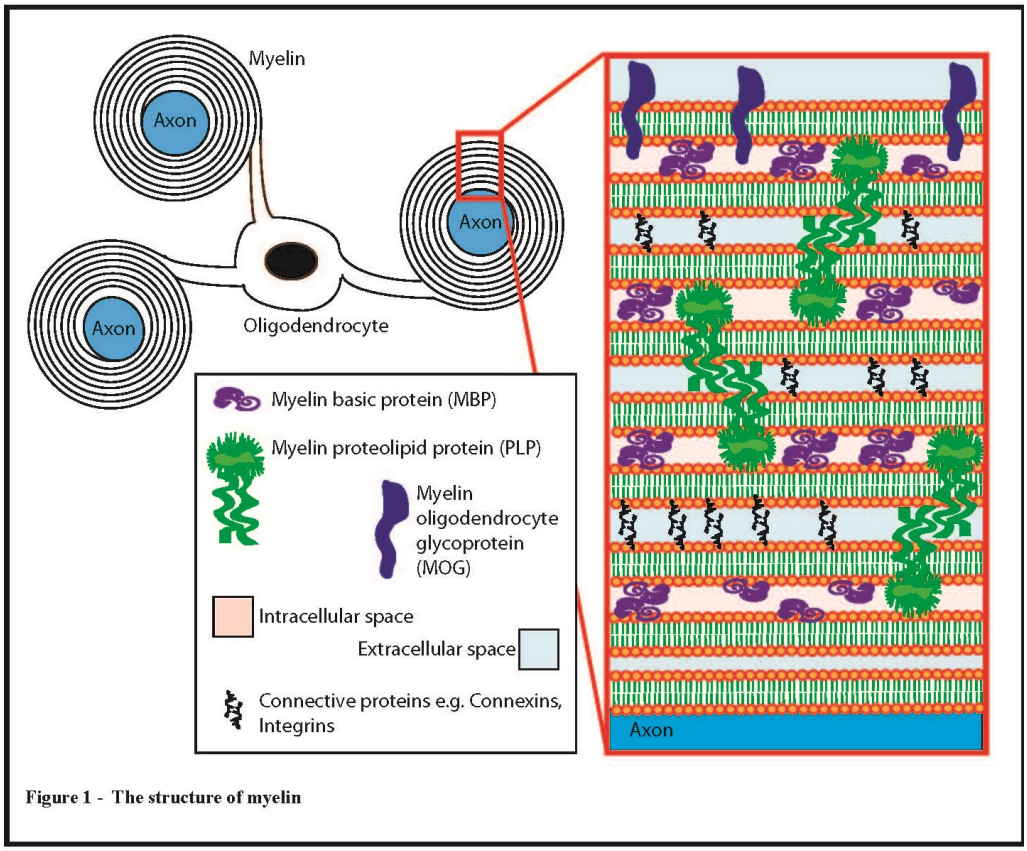

Figure 1 - The structure of myelin

than slightly less compact, but still fully functional, myelin [10]. This is in contrast to mutation [11-13] or over-expression [14,15] of PLP which is consistently detrimental in mice and humans. These studies seem to show that while MBP is a non-redundant component of myelin structure, other proteins are not essential for proper myelin sheath assembly.

1.1.2 Oligodendrocytes

Oligodendrocytes arise from oligodendrocyte precursor cells (OPCs) in the ventral ventricular zone of the developing brain and

spinal cord. Sonic hedgehog (Shh) signalling acts to pattern tissue in this area (for review see [16]) and initiates the expression of two transcription factors that direct cells to the oligodendrocyte lineage; Olig1 and Olig2 [17,18]. Ectopic expression of Shh elsewhere in the spinal cord leads to ectopic development of OPCs [19]. The expression of Olig1/2 in turn leads to the expression of other early glial cell lineage transcription factors such as Sox10 [20] and Nkx2.2 [21,22] which are required for differentiation of oligodendrocytes. OPCs were first identified as a population of bi-potential glial progenitors resident in the perinatal rat optic nerve, and so called O-2A cells for their ability to develop either into oligodendrocytes (O) or type-II astrocytes (2A) [23]. Later, so-called 'adult' O-2A cells were discovered in adult optic nerve, demonstrating that they persist into adult life in the mammalian CNS [24]. In the adult brain, these adult OPCs persist as a population of dividing cells that, despite only making up 5-8% of adult brain cells [25], represent 70% of proliferating cells as labelled by BrdU pulses in the adult brain [26]. They are identified by expression of the surface markers platelet derived growth factor receptor alpha (PDGFRa) and chondroitin sulphate proteoglycan (NG2). $NG2^+$ glial cells have many processes, which are highly branched, and are found in a continuous network throughout the CNS [27]. They express AMPA receptors, are capable of synaptic communication with neurons [28] and have been observed forming synapses with unmyelinated axons in the corpus callosum [29], although why they should be sensitive to axonal signalling is unclear. Although myelination occurs in late prenatal and early postnatal stages in mammalian brain, adult OPCs still generate oligodendrocytes in the adult CNS and studies have shown that 20% of oligodendrocytes are generated after 7 weeks of age in mice [30]. It has also been shown

that in models of CNS inflammation [31] and focal demyelination [32] OPCs can respond by proliferating and migrating to the damaged area before forming new oligodendrocytes, indicating a system of repair and regeneration.

1.1.3 Benefits of myelination

Myelin sheaths are separated from each other along an axon by small unmyelinated segments, known as Nodes of Ranvier. Oligodendrocytes induce the clustering of $Na_v1.2$ voltage-gated sodium channels (VGSCs) at these nodes [33] during development which are later replaced by $Na_v1.6$ VGSCs [34,35]. Clustering is dependent on an as-yet unidentified soluble factor [33]. Following experimental ablation of oligodendrocytes and demyelination, sodium channel clustering disappears within 1 week, whereas if viable oligodendrocytes remain in the vicinity, clustering can persist for up to 8 weeks [36]. This is probably due to the preservation of soluble signals from oligodendrocytes. Ultimately, restoration of myelin sheaths is required for nodal re-organisation, which shows similarities to developmental myelination in that $Na_v1.2$ expression precedes $Na_v1.6$ expression [36]. The surface of the axon located beneath a myelin sheath, or internodal region, is separated from the axonal surface at Nodes of Ranvier by the paranodes, assembled by transmembrane proteins such as CASPR, contactin and neurofascin 155, on the axonal and oligodendrocyte membranes, which form a diffusion barrier, and aid organisation of the Node [37] (for review see [38]). These proteins anchor the edges of the myelin sheath to the axonal membrane and maintain a high concentration of VGSCs in the node. This structural arrangement allows electrical insulation of the axon. The myelin sheath, with its multiple layers of membrane,

increases the capacitance of the axon to the extent that charge travels along the axon in a series of leaps between nodes rather than flowing through the axonal cytoplasm. This conduction via local circuits between nodes, known as saltatory conduction, is an order of magnitude faster than conduction through the axon itself. Following the destruction of myelin sheaths (demyelination), it has been shown that the regeneration of myelin sheaths around nerve axons, a process known as remyelination, can restore the conduction of electrical signals, by restoring the structural arrangement [39].

As well as allowing rapid nerve conduction, myelination by oligodendrocytes also provides trophic support to axons and protects against axonal degeneration. Two recent studies show that oligodendrocytes supply axons with energy in the form of lactate. The first study shows that oligodendrocytes shuttle lactate to axons through the myelin sheath [5]. The second shows that the lactate transporter monocarboxylate transporter 1 (MCT1) is enriched in oligodendrocytes, and that reducing the level of MCT1 translation in oligodendrocytes in vivo leads to axonal degeneration [40]. This lends support to the hypothesis that myelination or remyelination prevent axonal degeneration in demyelinating diseases such multiple sclerosis (MS). Evidence for this hypothesis includes the PLP^{null} mouse, in which oligodendrocytes are capable of forming apparently structurally-normal myelin, yet axonal degeneration is observed [41]. The same is true of the myelin-associated glycoprotein-null (MAG^{null}) mouse [42], despite this protein having a relatively minor role in the assembly of the myelin sheath. Finally, deletion of the oligodendrocyte population by expressing the diphtheria toxin (DT) receptor in PLP-expressing cells prior to DT application, also leads to axonal degeneration [43]. Taken together, these studies

suggest that the myelin sheath is required for axonal stability in vivo.

1.2 Multiple sclerosis

1.2.1 Symptoms and pathology

MS is an autoimmune neurodegenerative disorder that is the second most common cause of disability amongst young adults in the UK after traumatic injury [44]. It carries a lifetime risk of 1 in 400 and has an incidence of 120 per 100,000 people [45]. Scotland has the world's highest prevalence of MS with 1 in 500 people suffering from the disease [46]. Despite decades of research, the initial trigger for the disease is unclear although pathology is thought to be driven by self-reactive T lymphocytes accumulating in the CNS. Disease onset often occurs between 20 and 40 years of age and the most common symptoms of the disease are cognitive, motor and sensory defects. Disease course commonly takes one of three main patterns. The most common pattern of MS, affecting 80% of MS patients, is 'relapsing-remitting' MS. This consists of periods of 'relapses' with clinical symptoms followed by periods of almost complete recovery, termed 'remissions'. Many of these patients convert to 'secondary progressive' MS, where their symptoms gradually worsen even during remission despite few or no relapses. In 'primary progressive' MS, clinical symptoms become steadily worse over time without any relapses. It is thought that relapses are caused by episodes of transient inflammation of the CNS, with concomitant demyelination.

The pathology seen in MS consists of plaques or 'demyelinated lesions' in the white matter in the brain and spinal cord, often associated closely with blood vessels, with oligodendrocyte damage or destruction, demyelination and axon degeneration. The loss of the myelin sheath around axon processes leads to loss of nerve function in these areas and the associated symptoms of the disease. Pathologists categorise lesions according to levels of active inflammation, demyelination and regeneration. Lesions are commonly referred to by this method as chronic, active, chronic-active or 'remyelinated' in which myelin sheaths have been partly replaced. Initially, early in the course of MS, lesions repair and remyelinate, but over time this fails and the damage becomes chronic. It has been found that capacity for repair reduces with the age of the patient [47,48]. It has been hypothesised, on the basis of animal studies as described above where myelin is required for axonal health, that demyelination (and failure of remyelination) makes axons vulnerable to axonal degeneration. It is hard to prove a causative link from pathological study of MS tissue; however it is known that axonal damage is positively correlated with increasing disability [49]. Furthermore, in a mouse model of chronic CNS inflammation, experimental autoimmune encephalomyelitis (EAE), axonal degeneration is greatest in areas of acute demyelination and remyelinated areas have no significant axonal damage [50]. The most persuasive evidence that remyelination protects axons is a study in which mice were fed dietary cuprizone, a copper chelator which is toxic for oligodendrocytes, and axonal loss was quantified under different conditions. Mice were treated with cuprizone to globally demyelinate the CNS and either X-irradiated to kill OPCs and inhibit remyelination [51] or left to remyelinate normally. Normal remyelination resulted in reduced axonal loss in this model, albeit

in the absence of autoimmune inflammation [52]. These studies taken together suggest that remyelination is protective and that boosting the process of remyelination could be therapeutic in patients with MS.

1.2.2 Risk factors in MS

In pairs of monozygotic twins in which one twin suffers from MS the chance that the second twin will also develop MS is 26% [53]. This is in comparison to the risk for first-degree relatives of 3-4% and a general population risk of 0.1-0.2% [54], suggesting a significant contribution of genetic factors to lifetime risk. Further support for the heritability of MS risk comes from studies of adopted and biological siblings of MS sufferers. These show that having a sibling with MS increases lifetime risk in biological siblings whereas adopted siblings had a risk comparable to the general population, suggesting that genetic factors are more important than environmental factors [54]. Through GWAS studies, increasingly more gene loci have been discovered which associate with MS risk [55,56]. Almost all these genes are associated with the immune system, with the greatest risk imparted by alleles in the human leukocyte antigen (HLA) class II genes, located on chromosome 6 [57]. These genes code for antigen-presenting proteins that bind peptides within cells and travel to the cell surface where the peptides are displayed to immune cells. There are three classes of HLA class II protein complexes, named DP, DQ and DR, and each is a combination of two peptide chains. Due to the great diversity required to recognise potential threats, there is great variability in the HLA protein loci. Many HLA alleles are inherited together in haplotypes and it is the extended HLA 'DR15' haplotype of three

alleles - DRB1*1501, DQA1*0102 and DQB1*0602 - that carries the greatest risk of MS. Homozygosity for this haplotype increases lifetime risk six-fold [58]. Other haplotypes, such as DR3 and DR4, have been shown to increase risk but to a lesser extent than DR15 [59].

Epidemiological studies have also suggested a number of environmental factors that influence an individual's lifetime risk of MS. Among these, it has been shown that the incidence of MS appears to increase in temperate climates, with increased distance from the equator. Subjects moving to another climate earlier than, approximately, the age of 15, assume the lifetime risk of their new region. Those moving after 15 years maintain the risk from the place where they grew up; hinting that development early in life determines a subject's lifetime risk of MS [60]. Environmental factors such as diet are also thought to contribute to MS risk. In particular, vitamin D deficiency, possibly accounting for the increase in incidence at high latitudes due to lower UV radiation levels [60], has been put forward as an important risk factor. A recent study showing that mutations in a protein involved in vitamin-D metabolism increase MS risk provides strong evidence in support of this association. In a cohort of Canadian MS patients, with a strong history of MS in the family, a variant in the CYP27B1 gene was enriched. This variant causes inactivation of 25-hydroxyvitamin D-1α hydroxylase, which converts 25-hydroxyvitamin D-1α to active vitamin D, leading to vitamin D deficiency [61]. Individuals who are homozygous for this rare allele suffer from a genetic form of the bone disease Rickets, whereas the study found that heterozygous individuals had an increased risk of developing MS. Studies aiming to correlate lifetime vitamin D

levels with the chance of being diagnosed with MS also provide evidence for a link. In a study including a total of 182,000 nurses, the consumption of supplemental vitamin D was correlated with a 40% lower risk of developing MS [62]. In another study, the health records of active US army personnel were used to estimate each individual's serum vitamin D levels. Over the course of the study, those in the highest quintile of vitamin D levels were 62% less likely to develop MS than those in the lowest [63]. The growing number of similar studies that associate vitamin D levels with decreased risk of MS has led to calls for mandatory dietary vitamin D supplementation in at-risk populations [57].

The greatest, and perhaps most well-known, risk factor for MS is infection with Epstein-Barr virus (EBV), which causes glandular fever. In most people, infection is at an early age and asymptomatic. However, some adults survive to adulthood with negative serum titres of immunoglobulin G (IgG) antibodies against the latent EBV protein anti-EBV nuclear antigen 1 (EBNA-1), which is the main marker for immunity to EBV. First infection at adolescence or adulthood can cause a more serious disease, infectious mononucleosis (often referred to as 'Mono'), which has a number of symptoms including high fever, fatigue and lymphadenopathy[57]. Among those who have a medical history of infectious mononucleosis, who are assumed to have avoided exposure to EBV in childhood, the incidence of MS was increased by two to three times [64]. Another study of more than 8 million US army recruits showed that only 5% were negative for EBV antibodies when they were recruited. Of this cohort, 10 later developed MS and all of these individuals had suffered from infectious mononucleosis a few months prior to developing MS [65].

This study supports the hypothesis that primary exposure to EBV relatively late in life may significantly increase MS risk. EBV infects B-cells and has modulatory effects on the immune system and this has been suggested as a possible mechanism for any causative role in MS.

1.2.3 Experimental models of Multiple Sclerosis

The chronic nature of the disease and complex interaction of the adaptive immune and nervous systems over time have made MS a difficult disease to model, especially as there is no natural animal model of the disease. There are good model systems that can recapitulate the various immune and degenerative aspects of MS but not usually in combination. The most commonly used animal models of MS can be divided into predominantly immune-mediated and toxin-induced which each have their advantages and disadvantages in different situations depending on the research question.

Experimental autoimmune encephalomyelitis

This is an autoimmune disease of mice initiated by immunisation with one of a number of myelin proteins. First developed in primates [66], EAE is commonly induced with MBP and MOG peptides emulsified in adjuvant, such as complete Freund's adjuvant (CFA). This provides the inflammatory insult required to prime the immune system, allowing T cells that are specific for the particular peptide used to be activated and target myelin sheaths. This model may accurately recapitulate the CNS inflammation typical of MS and can produce a disease course with a monophasic

course (similar to a relapse), or several relapses, or even, with the Biozzi mouse model, a progressive neurodegenerative phase, depending on the experimental induction protocol and the strain or species of animal used. EAE may be very useful for analysing the immune responses that take place in CNS autoimmune disease and has been used to demonstrate both the requirement for Treg in resolving CNS autoimmune inflammation [67] and that injection of Tg4 Treg specific for the Ac1-9 MBP peptide (see methods) can ameliorate EAE induced with the same peptide and can help to reduce the recovery time in EAE induced with a peptide from a different myelin protein (PLP) [68]. While EAE is obviously very good at examining the roles that different T cell types play in development, maintenance and recovery of CNS autoimmune inflammation, it does not, for the most part, model the process of remyelination as well. Therefore, in order to study remyelination, we must use a different model, one that does not have the added variable of CNS inflammation.

Cuprizone-induced demyelination

One aspect of MS that is not modelled well in EAE is the re-myelination of demyelinated axons following oligodendrocyte death. This process restores the myelin sheath to axons and facilitates recovery. There are several ways of modelling this behaviour experimentally. One method is by using the Cuprizone model of demyelination. Cuprizone is a copper chelator that causes widespread death of mature oligodendrocytes when fed orally to 8-week old mice. The selectivity for oligodendrocytes is thought to be a result of the enormous metabolic demands placed on these cells by the formation and maintenance of myelin sheaths and this makes them particularly vulnerable to the effects of this toxin.

Cuprizone administered orally over 1-2 weeks effectively demyelinates the CNS and, following removal of the toxin from the diet of the mouse, remyelination begins after around 4 days [69,70]. This model effectively models demyelination and subsequent remyelination, but the insult occurs throughout the CNS rather than in the distinct lesions observed in chronic MS, and there is some toxic axonal damage noted as well.

Lysophosphatidyl choline-induced white matter lesions

Lysophosphatidyl choline (LPC), also known as Lysolecithin, is used to produce focal demyelinated lesions. LPC is a detergent molecule that emulsifies lipids and therefore causes damage to phospholipid membranes [71]. The huge surface membrane area to cell body volume ratio of oligodendrocytes ensures that at low doses this toxin preferentially targets these cells, with little non-specific damage to surrounding cells. In vivo, these lesions are induced through stereotaxic injection of LPC into a specific area of the CNS, causing localised demyelination and inflammation. In rodents these lesions fully remyelinate over the course of four weeks and can be used to model the demyelination and remyelination in focal lesions without the complication of an on-going immune reaction and inflammation (as in EAE). It is also possible to apply LPC to ex vivo slices of mouse brain, producing a useful in vitro model of demyelinated tissue.

All of these systems model specific aspects of MS pathology but fall short of modelling the disease as a whole. As such they complement each other but more chronic variants of these experiments would be extremely useful for probing the chronic pathology of MS. I have chosen to use LPC lesions for the in vivo

experiments in this project as this will allow examination of how Treg influence remyelination in the absence of neural inflammation, a variable that I wish to exclude in order to test the direct effects of Treg on oligodendroglial cells rather than indirect effects on CNS inflammation, which may also be beneficial for remyelination.

1.3 Remyelination

1.3.1 Oligodendrocyte precursor cells

Remyelination is the regenerative process, known since the early 1960s [72], through which missing or damaged myelin sheaths are replaced by OPCs [73]. These OPCs are multipotent progenitor cells found throughout the CNS that are able to proliferate and migrate and can differentiate into mature myelin-forming oligodendrocytes, as well as astrocytes [31] and they have been reported to occasionally form Schwann cells [32] and neurons [30,31]. OPCs are identified through the expression of the cell surface marker proteins NG2 (a chondroitin sulphate proteoglycan) and alpha-type platelet-derived growth factor receptor (pdgfrα) [74]. As they develop from simple uni- or bi-polar OPCs, they successively express the marker proteins NG2, O4 sulphatide (O4) and finally MBP as they mature into post-mitotic oligodendrocytes (Fig. 2) that are typically highly branched with many membranous processes. These processes contact demyelinated axons and extend a sheath around the axon that is subsequently filled with myelin proteins and extends laterally along the axonal membrane. This process is distinct from

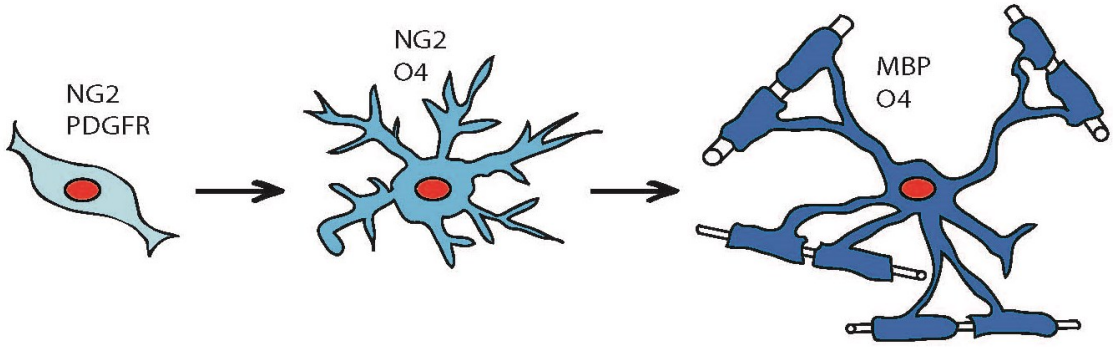

Figure 2 - The three main stages of oligodendrocyte differentiation.

NG2 - chondroitin sulphate, PDGFR - platelet derived growth factor receptor, O4 - sulphatide, MBP - myelin basic protein.

developmental myelination in that it occurs against a background of oligodendrocyte cell death that has left an axon without a myelin sheath and in an inflammatory environment. In order for each OPC to successfully remyelinate a demyelinated axon it must first survive the initial insult, then migrate to the site of injury, proliferate and finally differentiate from a small, mobile OPC into a large membranous oligodendrocyte: a process requiring huge amounts of protein synthesis and energy expenditure.

1.3.2 Challenges to remyelination

Remyelination is inefficient In MS patients: not all lesions remyelinate equally. In some patients, remyelination can be relatively complete and relatively poor in others [75]. A number of theories have been put forward as an explanation for this including depletion of OPCs from lesioned areas, the stalling of OPC differentiation and OPC migration failure (for review see [76]). In rodents, at least, OPCs effectively repopulate areas that have been depleted of OPCs in vivo [77] and repeated demyelination of the

same area does not affect the frequency of OPCs in the lesioned area long-term and does not prevent subsequent remyelination [78]. In contrast, a failure of differentiation as a remyelination-blocking mechanism is supported by studies of lesions, some of which show a sufficiency of OPCs that apparently fail to differentiate [79]. A possible explanation for this is that the environment of the lesion is not supportive of proper oligodendrocyte differentiation. Often, as in MS, tissue damage and inflammation produce a complex milieu of proteins in the environment around the site of damage that can either enhance or inhibit remyelination. For example it has been shown that mice lacking tumour necrosis factor-alpha ($TNF\alpha^{-/-}$) have much slower remyelination in the CNS. In these $TNF\alpha^{-/-}$ mice 78.4% of axons in the corpus callosum remain demyelinated 2 weeks after a demyelinating 'cuprizone' diet is withdrawn, compared to 11% in wild-type controls [2]. Many other cytokines including interleukin-2 (IL-2) [80] and interferon-gamma (IFNγ) [81] have been shown to have an effect on oligodendroglial cells, often inhibitory (see Table 1). An added challenge is the finding that, in rats at least, greater age decreases the ability of OPCs to remyelinate axons [82]. This is due both to deficits in differentiation and in recruitment to damaged areas [83]. This is consistent with research that shows that remyelination of MS lesions is less complete in autopsy tissue from patients with chronic MS, who are older, compared with tissue from diagnostic biopsies of newly-diagnosed MS, who are younger [84]. Inhibition of remyelination-promoting proteins by age-related DNA changes has also been implicated in decreasing remyelination efficiency with age [85]. Recent studies also suggest that excess hyaluronan in lesion sites blocks OPC maturation. Examining lesions from patients suffering from MS and mice with EAE shows that astrocytes secrete

hyaluronan that builds up in lesions and inhibits OPC differentiation [86]. Further work has shown that hyaluronidases expressed by oligodendrocytes first digest high molecular weight (HMW) hyaluronan and that low molecular weight (LMW) hyaluronan is the molecule that blocks differentiation, acting through toll-like receptor 2 (TLR2) [87]. The protein LINGO-1 has also been shown to inhibit remyelination in lesions. Antibodies blocking this protein boost remyelination in a rodent model of focal demyelination [88]. The use of these antibodies has also been suggested as a therapy for MS in humans [89]. Myelin debris has also been shown to inhibit oligodendrocyte maturation [90], further establishing that demyelinated lesions are often not permissive to repair.

1.3.3 Histological markers of remyelination

The histological markers of remyelinated axons are typically thinner than expected myelin sheaths and shorter internodes. This can be seen on sections of lesioned CNS tissue stained with a luxol fast-blue (LFB) stain. Remyelinated lesions stain blue, just as normal white matter does, however the shade of blue is paler, which is a sign of reduced myelin sheath thickness. These plaques are known as 'shadow plaques' as a result. Myelin sheath thickness in a healthy adult conforms to a tightly controlled relationship between axon diameter and myelin thickness, described by the 'G ratio': the ratio of the diameter of the axon alone to the total diameter of the axon and myelin sheath. In the human nervous system, myelin thickness increases with axon diameter in a proportional relationship. It has been suggested that a value of 0.6 is the theoretical optimum value of axon diameter to myelin

thickness in the healthy nerve [91]. The actual observed ratio is between 0.76 and 0.81 [92]. Remyelinated nerves naturally have a higher than expected G-ratio value due to reduced myelin sheath thickness. As well as thinner myelin sheaths, remyelinated nerves have shorter than average internodes. However, even with thin myelin sheaths and short internodes, remyelinated axons regain sufficiently fast conduction velocity to allow normal function in MS patients or in animal models of MS. As a result, the enhancement of remyelination has been identified as a key therapeutic strategy in MS and is the focus of much research.

1.4 T lymphocytes

1.4.1 T cell activation

It is believed that the pathology of MS is produced by the activation of T helper cells, also known as T effector cells (Teffs), which have specificity for peptide sequences found in myelin proteins and launch autoimmune attacks against cells displaying these proteins. The activation of T cells requires simultaneous binding of a T cell receptor (TCR) by its cognate peptide and ligand binding of co-stimulatory surface proteins such as CD80. This co-stimulation limits the activation of T cells to sites where inflammation, which causes antigen-presenting cells (APCs) to up-regulate the surface expression of co-stimulatory molecules, coincides with presentation by major histocompatibility complex (MHC) molecules of antigens for which TCRs on the T cell are specific. In MS, host proteins are processed and displayed as peptides on APCs, and T cells may mount an immune response against cells and tissues containing these proteins – an autoimmune

response. It is well established that T cell clones that recognise epitopes of myelin proteins exist in MS patients [93], which supports this hypothesis. However, no specific host protein has been identified in all or even subsets of MS patients, and so the target of the autoimmune response remains unknown.

1.4.2 T helper cells

Two types of T helper cells have been implicated in the pathology of MS; these are T helper 1 (Th1) and the more recently characterised T helper 17 (Th17) cells [94,95]. Th1 cells are predominantly pro-inflammatory T cells that are characterised by the expression and secretion of the pro-inflammatory cytokine IFNγ. Th17 cells, on the other hand, produce a different set of cytokines. Studies have shown that these cells, named for their distinctive production of the cytokine interleukin-17 (IL-17), co-operate with Th1 cells in producing the CNS inflammation that leads to oligodendrocyte cell death and loss of myelin in MS. It was thought for a long time that IFNγ was the main driver of the CNS inflammation typical of MS. However, in the last twenty years several other cytokines have been identified that are necessary for induction of the murine MS model EAE. In experiments with mice in which the cytokines are genetically deleted singly it has been shown that interleukin-6 (IL-6) and interleukin-23 (IL-23) are required for EAE induction with an immunodominant MOG epitope. This is thought to be caused, in the case of IL-$6^{-/-}$ mice, by suboptimal activation of T cells and in IL-$23^{-/-}$ mice by poor differentiation of naïve T cells into Th17 cells [96-98]. More recently a third cytokine has been added to the list of cytokines that are 'non-redundant' in EAE. Two groups, reporting in Nature Immunology

recently, have shown that the encephalitogenicity of Th17 cells is dependent on the expression of GM-CSF, a cytokine secreted by the T cells themselves [99,100]. In the proposed model, Th17 cells secrete GM-CSF that stimulates the secretion of IL-23 by dendritic cells (DCs), highly potent APCs. IL-23 in turn induces Th17 cells to produce more GM-CSF in a positive feedback mechanism that may explain the chronic nature of Th17-mediated immune reactions. This model also elegantly explains the importance of IL-23 in producing EAE. How GM-CSF produces EAE pathology is currently unclear although one proposed mechanism is through stimulation of a small number of $CD11b^{hi}$–expressing myeloid cells [100] or through macrophages [99]. However, whilst these subtypes of T cell are highly damaging to the CNS there are other types of immune cell that work to limit their activation and thereby restrict the damage caused by these cells.

1.4.3 Central tolerance: negative selection

During normal thymic development of T lymphocytes, those cells that bind strongly to 'self' peptides (i.e. self-reactive) are deleted through a process called activation induced cell death (AICD). This mechanism, known as "Central Tolerance", eliminates cells expressing self-reactive TCRs that arise naturally through VDJ recombination. In AICD, APCs in the thymus present self-peptides to developing T lymphocytes ectopically through the actions of the autoimmune regulator (Aire) transcription factor [101]. Those cells that bind strongly to proteins displayed on APCs, such as macrophages and dendritic cells, undergo apoptosis. This essential mechanism prevents strongly self-reactive cells from leaving the

thymus and stimulating autoimmune attacks against body tissues. Humans and mice with a mutation causing dysfunction in the Aire protein suffer from severe autoimmune reactions as central tolerance fails [102,103]. In humans this disease is known as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) [104] and is nearly always fatal. However, even in healthy people, some T cells expressing weakly self-reactive TCRs are allowed to develop normally without deletion. These cells with weakly self-reactive TCRs also express TCRs with specificities to a large number of non-self antigens and therefore effectively fight pathogenic infection. Removing them would compromise host immunity and be deleterious to the organism. So organisms that possess an adaptive immune system, such as humans and mice, have a circulating population of T-cells that also have the potential to launch an autoimmune attack on host tissues. This was shown in experiments that deleted a defined population of $CD4^+$ cells in mice, producing autoimmune disease phenotypes [105].

1.4.4 Regulatory T cells

The evolved solution to this compromise is the presence of a population of self-regulating T cells known as Regulatory T cells (Treg) which suppress autoreactive T cells. These Treg also express host-specific TCRs and are more reactive to self-antigen than the weakly self-reactive Teffs [106,107]. The difference is that the increased 'strength' of self-reactivity exhibited by these cells in response to DC-expressed self-antigens in the thymus causes these cells to be sent down a different developmental pathway. Expression of the transcription factor forkhead box protein 3 (foxp3) is induced and

these cells develop a 'suppressor' phenotype. Foxp3 expression is induced by a number of transcription factors including the transcription factors Foxo1 and Foxo3 [108] and it itself controls the transcription of several genes that are vital for Treg function, such as IRF4 and Blimp-1 [109]. Treg leave the thymus and circulate throughout the body binding to antigen-presenting cells (APCs) that express their cognate self-antigen bound to MHC proteins on their surface. On binding to these MHC-bound peptides, which are also bound by weakly self-reactive T cells, Treg become activated. This leads to increased secretion of the suppressor cytokines interleukin-10 (IL-10) and transforming growth factor β (TGFβ). It also leads to enhanced expression of certain surface proteins such as CTLA4, thought to act as a negative regulator of T cell activation through the trans-endocytosis of co-stimulatory molecules expressed on APCs [110]. IL-10 has been shown to inhibit the activation of Th17 cells [111,112] that are associated with chronic autoimmune attacks whereas TGFβ is known to stimulate the development of new Treg, possibly increasing the effective local Treg population and it is also known to protect Treg from apoptosis during their development in the Thymus [113]. In response to activation, Treg also secrete IL-2, a cytokine that is necessary for their development and proliferation. This may also lead to an increase in the local population of Treg, leading to more effective suppression of self-reactive T cell activation. This so-called 'Peripheral Tolerance' acts to suppress the activation of T helper cells in the immediate vicinity and quell autoimmune attacks by self-reactive T cells. So vital is this regulation that mice and humans with deleterious mutations in the foxp3 transcription factor suffer from fatal autoimmunity similar to pathology associated with Aire defects [105,114].

1.4.5 Subtypes of Regulatory T cells

Treg come in several different types and are part of a wider family of immune cells thought to have suppressor function. Of the three described sub-groups of Treg, two are found in vivo and the third can be produced from cells that are $CD4^+$ $foxp3^-$ in vitro. The first group are known as 'natural' Treg (nTreg) since they arise in the thymus and cells of this subtype are produced by the thymus throughout life. These cells are the only known 'dedicated' foxp3-expressing suppressor cells. These cells were first described in 1995 as $CD4^+$ $CD25^+$ suppressor cells that were capable of rescuing 'nude' mice reconstituted only with $CD4^+$ $CD25^-$ cells from developing fatal autoimmune disease [105,114]. It was not until more recently [115] that mutations of the transcription factor foxp3 were found to be the cause of immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), a severe and often fatal autoimmune disorder of humans characterised by multiple simultaneous autoimmune reactions against body tissues. This led to the discovery that foxp3 expression is essential for the regulatory phenotype of Treg and transgenic mice with fluorescent markers driven by the foxp3 promoter have made it much easier to obtain and identify Treg from these mice. Natural Treg typically secrete TGFβ and IL-10 and have a stable phenotype. The second type of Treg, sometimes referred to as 'adaptive' or 'adapted' Treg (aTreg) arise from $CD4^+$ $foxp3^-$ T cells in vivo in response to environmental cues such as high concentrations of TGFβ. These cells have a transient suppressor phenotype and may be important in increasing the

frequency of Treg cells locally in response to signals from recruited nTreg, helping to control nascent autoimmune reactions. The third subtype of Treg is the 'induced' Treg (iTreg). These cells represent a therapeutically relevant class of Treg made in vitro from $CD4^+$ $foxp3^-$ cells. This technique was first described in 2003 by Chen et al. [116] who showed that application of TGFβ and simultaneous stimulation of both the TCR and a separate surface receptor, CD28, could turn otherwise non-suppressing T cells into Treg in vitro through the induction of foxp3 expression in naïve T cells. Since then several groups have published refinements to this technique [117] culminating with the discovery by Davidson et al. in 2007 that IL-2 is required for the efficient induction of iTreg cells [118]. This discovery has brought the possibility of using Treg as an immune therapy against a number of autoimmune diseases a step closer. In the mammalian immune system, $foxp3^+$ nTreg form less than 10% of the circulating $CD4^+$ T cell population. Even if these cells could be isolated from patients without compromising Treg surveillance it would be too few cells to provide effective therapy. On the other hand iTreg can be made from the other 90% of the $CD4^+$ cell population and therefore may provide enough cells for therapy. Recent breakthroughs in expanding Treg in the lab have brought this possibility a step closer [119-122]. Several clinical trials are on-going using injections of nTreg to protect against autoimmune diseases such as Graft Versus Host Disease (GVHD)[119,123,124]. Some of these trials use polyclonal Treg, meaning Treg that do not express TCRs with a specific affinity for the affected tissue. Some mouse studies have used Treg from transgenic mice that express a TCR specific for a particular protein epitope, such as the MBP-specific Tg4 mouse [125] or the ovalbumin-specific OT-II mouse [126], on all T cells. These cells are described as monoclonal, as the whole

population has specificity for the protein of interest. In humans, monoclonal populations can be produced by selecting iTreg clones with a known specificity and encouraging these cells to proliferate, however this approach is expensive and therefore some groups have elected to use polyclonal cells with more general non-specific activation to the tissue of interest during autoimmune inflammation. The efficacy of monoclonal Treg in controlling CNS inflammation has been demonstrated in the mouse model EAE. In MS, Treg deficiency of numbers or function has been put forward by some as a possible contributory factor in the initiation of the disease [127,128]. It has also been suggested that Treg from MS patients are not as efficient at migration [129] and, further, that it is only a subset of Treg which express IL-7 receptor alpha-chain which are functionally impaired and that the rest are normal [130]. Treg have also been found within MS lesions seen in diagnostic tissue biopsies by some groups [1], although this is disputed by others [131]. In the context of murine EAE, Treg are routinely found in the CNS at the peak of disease and express a particular cytokine, Leukaemia inhibitory factor (LIF), at levels that are almost seven-fold higher than similar cells in the periphery (unpublished data from the Anderton lab).

1.5 Leukemia inhibitory factor (LIF)

1.5.1 LIF signalling

Structurally, LIF is 179 amino acids long and the murine form has 78% homology with the human LIF protein [132]. The tertiary structure of LIF is a bundle of four alpha helices in an 'up-up-

down-down' configuration [133] (Fig.3(1)). The LIF protein is a member of the glycoprotein 130 (gp130) family of cytokines and, as such, acts through a heterodimeric LIF receptor (LIFR) made from the gp130 and LIF receptor β (LIFRβ) subunits [134]. Ablation of the LIF gene is not lethal in mice and the only major phenotype is female infertility [135] that can be rescued with LIF injections [136]. On the other hand, loss of LIFR signalling results in loss of motor neurons [137]. Following LIF binding to LIFRβ (Fig.3(2)), the complex dimerises with gp130 and is phosphorylated by the Janus kinases 1 and 2 (Jak1, Jak2) (Fig.3(3)), which in turn phosphorylate the transcription factor STAT3. Phosphorylation of STAT3 induces its dimerisation (Fig.3(4)) and the STAT3 dimer then translocates to the nucleus where it can either bind to DNA sequences containing a $TT(N)_5AA$ motif or form transcriptional complexes with other factors and bind to $TT(N)_6AA$ motifs [138] (Fig.3(5)). Inhibitors of LIF function include the STAT-induced suppressor of cytokine signalling 3 (SOCS3), which forms an intra-cellular negative feedback loop (Fig.3(6)), and LIF binding protein (LBP), which is found extracellularly [139]. LBP is thought to act systematically and limit the effects of LIF to areas close to localised expression [140].

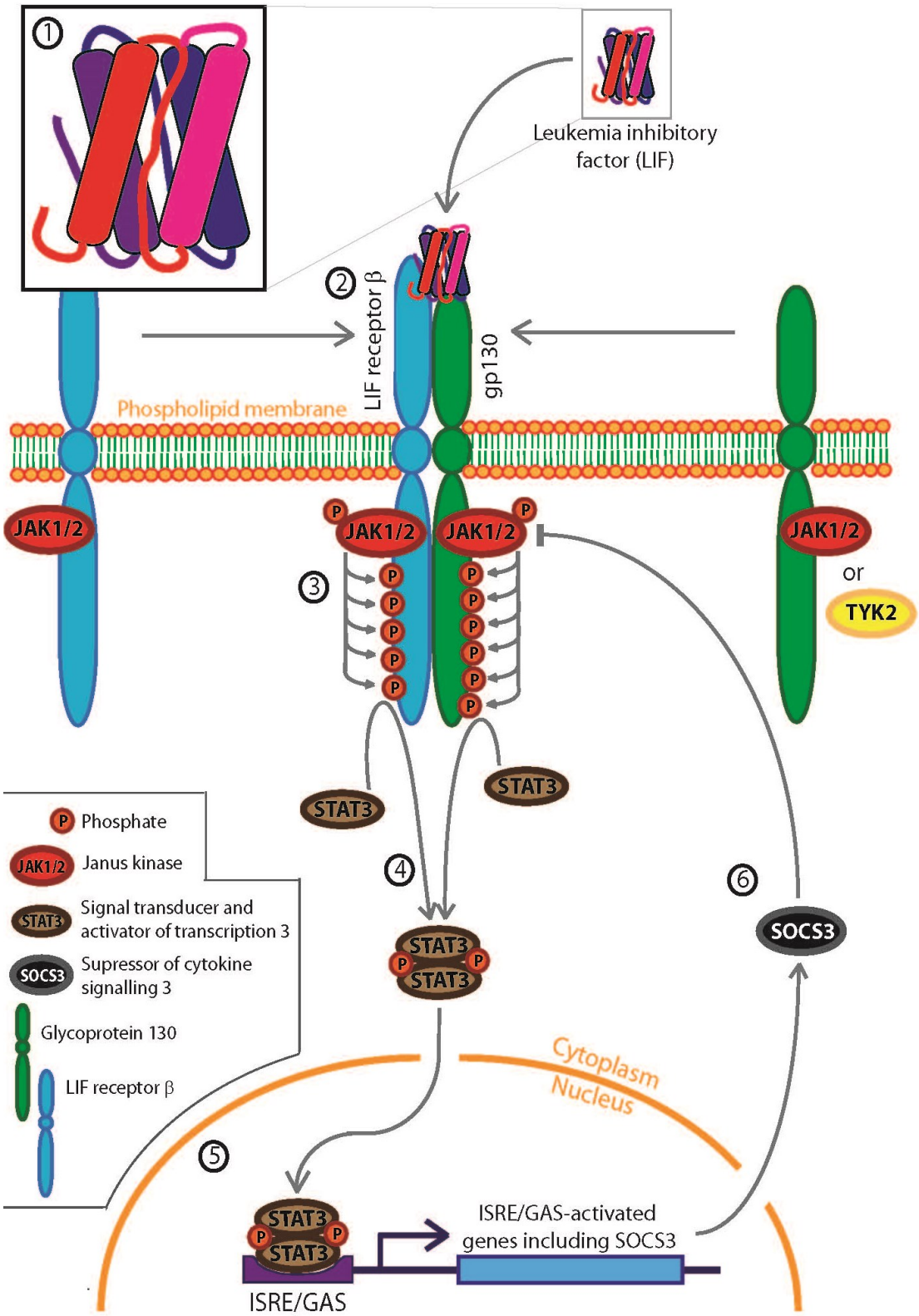

Figure 3 - The leukaemia Inhibitory Factor (LIF) intracellular signalling pathway

TYK2 - tyrosine kinase 2, ISRE - interferon stimulated response element, GAS - gamma activated sequence.

1.5.2 Function in CNS inflammation

First characterised in 1988 as a factor that stimulates the differentiation of the leukemic M1 cell line [141], it was quickly shown that LIF is capable of inhibiting the differentiation of embryonic stem cells [142-144]. In mice, LIF is a pleiotropic cytokine that acts on cells in many tissues, including the kidneys, the pituitary, the muscles and the bones [140]. During CNS inflammation, LIF is protective against demyelination and axonal degeneration. Using the murine model of autoimmune CNS inflammation, EAE, it has been shown that administering antibodies against endogenous LIF leads to more severe disease, reduces the number of oligodendrocytes in the spinal column and also reduces global expression of MBP [145]. This built on previous work showing that injections of recombinant LIF could decrease the severity of EAE in mice [146]. This holds true even when LIF is overexpressed only in the CNS [147], supporting a CNS-intrinsic protective mechanism, rather than peripheral immune suppression. Knocking out the LIF gene in mice also lessens inflammation and reduces early neutrophil infiltration of the CNS [148]. Further work suggests that this beneficial effect is independent of oligodendrocyte signalling as selectively knocking out gp130 expression in oligodendrocytes had no effect on the course of EAE [149]. Such manipulations would, however, be expected to knock out the interleukin-6 (IL-6), oncostatin M (OSM) and cardiotrophin 1 (CT-1) signalling pathways as well [150], which makes this result hard to interpret. LIF is also secreted by myelin-reactive T lymphocytes in the CNS of MS patients, and immunohistochemical staining shows that T cells in lesions also express LIF [151].

1.5.3 Function in oligodendrocytes

LIF also exerts its effects on oligodendrocytes. Several studies have shown that LIF is effective in inducing oligodendrocyte survival and maturation in vitro [152,153]. In co-cultures of neurons, astrocytes and oligodendrocytes, LIF is known to be secreted by astrocytes in response to neuronal activity. This secretion is enough to increase the number of mature MOG^+ oligodendrocytes in these cultures, an effect that is blocked by anti-LIF antibodies [154]. In the cuprizone-induced demyelination model in mice, LIF has also been shown to be beneficial. When treated with 50ug/kg of recombinant LIF, wildtype mice suffered less severe demyelination following cuprizone treatment. It was also found that $LIF^{-/-}$ mice suffer worse demyelination than wildtype mice [155], suggesting that LIF protects against demyelination. Furthermore, while administering 50ug/kg recombinant LIF during remyelination did not appear to have a beneficial effect, remyelination is inhibited in $LIF^{-/-}$ mice [155]. Delivering LIF to the CNS via adenoviral delivery has also been shown to increase OPC and microglia proliferation and the rate of hippocampal remyelination following cuprizone treatment [156]. It has also been shown that conditionally deleting SOCS3 in oligodendrocytes gave greater protection against cuprizone demyelination [157]. Taken together with more recent work from the same group [149], this suggests that LIF promotes the survival, proliferation and maturation of oligodendrocytes, but also protects axons from inflammation independently of oligodendrocytes: both attractive goals for MS therapeutics.

Therefore the beneficial effects of LIF have been demonstrated in the context of global CNS inflammation and global CNS demyelination, but this has not yet been tested in focal demyelination, which I sought to do.

1.6 LIF-containing nanoparticles

1.6.1 Advantages of using nanoparticles

Nanoparticles are tiny particles, often made from polymers of organic compounds, which are between 1 and 10,000 nanometres in diameter. They have several properties that are attractive to medical biologists. Firstly, they have a large surface area to volume ratio; a microgram of $1nm^3$ nanoparticles has the same surface area as 1kg of $1mm^3$ particles. They can also be manufactured in such a way that other compounds or molecules can be carried inside, and, by using particular compounds for their construction, they can also be made to biodegrade over a controllable number of days. The nanoparticles we have access to have all of these properties; they are roughly 100nm in diameter, they carry recombinant LIF protein within their structure, they are coated with avidin to provide binding sites for biotinylated antibodies and they biodegrade, when exposed to water, over seven days. The nanoparticles are made from a polymer, poly-lysine co-glycolic acid (PLGA), which is formed from two monomers: poly-lysine and glycolic acid. The relative amounts of these two molecules affect the rate at which these particles degrade [158]. In experiments intended to establish the immunoreactivity of these nanoparticles in vivo, very little reaction is detected. After being injected into tissue, after only a few hours,

microglial activation is observed but no T lymphocyte infiltration is detected over the entire time that it takes for these nanoparticles to degrade naturally [159]. Therefore, nanoparticles are attractive as carriers of therapeutic proteins.

1.6.2 Delivery and Construction

By binding avidin to palmitic acid molecules it has recently become possible to coat PLGA nanoparticles with avidin and thus make coating these nanoparticles with targeting antibodies straightforward [160]. This has recently been demonstrated by targeting LIF-containing nanoparticles to T lymphocytes with a biotinylated anti-CD4 antibody [161]. Targeting nanoparticles in this way has great utility, especially for intravenous injection. It is not clear whether nanoparticles of different sizes can cross the blood-brain-barrier (BBB) following intravenous delivery. A recent paper which used nanoparticles to deliver anti-inflammatory drugs to the brain across the BBB is an important proof-of-principle [162], but it is not clear whether bigger nanoparticles of more than 100nm in diameter can do this. With a view to future use in patients with MS, it may be that the disruption of the BBB which occurs in the disease is sufficient to allow access to nanoparticles of this size. Nanoparticles containing dopamine have also been implanted directly into the rodent brain with some success, as the growth of dopaminergic neurons is boosted in such experiments [163,164]. The PLGA-based nanoparticles used in my experiments are manufactured by initially mixing the murine LIF and PLGA together before immersing in oil. The oil immersion creates an emulsion of polymer and oil from which the nano-scale polymer

particles can be extracted and washed. Manufacturing these nanoparticles in this way ensures that LIF is encapsulated throughout the particle, ensuring that the protein is released over a longer time period. In fact these nanoparticles release LIF over 7 days, although most of the release occurs over 48 hours [161]. This construction method also creates a reasonably well-controlled size distribution of nanoparticle, with particles ranging between 50 and 200nm [161].

This project aimed to investigate the effect of Treg soluble cytokines, including LIF, on oligodendroglial cell biology, with the hypothesis that these cytokines may directly influence this biology and aid regeneration of myelin sheaths in pathology.

Chapter 2: General Tools and Methods

Animal work was carried out in accordance with the University of Edinburgh regulations under Home Office rules, with local ethical committee consent. Experiments were carried out under the personal license 60/12865 and project licenses 60/3879 and 60/4116.

2.1 Animals

I used CD1 mice (Charles River) for all OPC isolations using Treg. For the isolation of $CD4^+$ $foxp3^+$ and $CD4^+$ $foxp3^-$ cells a C57BL/6.$Foxp3^{GFP}$ mouse strain is used that expresses a fully functional fusion protein in which the eGFP gene is inserted into the first coding exon of the foxp3 gene [165]. I also used Tg4 mice, which express a transgenic TCR protein that is specific for the acetylated N-terminal Ac1-9 epitope of MBP (Ac-ASQKRPSQR) [125]. For LIF experiments, I used wildtype Sprague-Dawley rats as a source of OPCs. All animals were bred and maintained under veterinary supervision and in accordance with Home Office rules at a secure facility at the University of Edinburgh.

2.2 Primary OPC culture

The brains of P0-P2 rodent pups are harvested and the meninges are removed. The tissue is mechanically dissociated with scissors and then chemically dissociated with papain to obtain a suspension of cells in Dulbeccos modified Eagle medium (DMEM) (Gibco #41966). Cells are plated in T75 tissue culture flasks (Falcon #353110) in DMEM (Gibco #41966) supplemented with 10% b/v heat-inactivated Horse Serum (HiHS) (Gibco #26050) and 1% b/v penicillin and streptomycin (p/s) (Gibco #15140). After incubation

for 7-10 days in 5% CO_2 at 37°C, cultures are shaken and differential adhesion allows for the separation of OPCs from microglia and astrocytes as previously described [166]. Isolated OPCs are plated at 20,000 cells/well in 8-well chamber slides (Lab Tek #177445) in SATO medium [167,168] supplemented with 0.5% HiHS (Gibco #26050) and 10ng/ml fibroblast growth factor (FGF) (PeproTech #100-18B) and platelet-derived growth factor (PDGF) (PeproTech #100-13A) to maintain the OPCs in a proliferative, undifferentiated state. OPCs are cultured in these conditions for 24 hours before experimental factors are added. For (24hr) cell survival and differentiation experiments, conditioned media from other cell types are added at a 1:1 ratio with 2X concentrated SATO medium in the absence of growth factors. Following the addition of experimental factors, OPCs are cultured for either 24 or 72 hours before fixation with 4% paraformaldehyde (PFA) (Sigma-Aldrich #P6148) and staining. In cell survival experiments taking place over 72hrs, OPCs are plated in 1X SATO medium without growth factors until the 48-hour time point at which stage the experimental factors are then added as described previously. For proliferation experiments, experimental factors are added along with 5uM EdU (Invitrogen #C10340) and the cells fixed after 24hrs with 4% PFA (Sigma-Aldrich #P6148).

2.3 OPC Immunocytology

Cells are typically permeabilised using a 0.2% solution of Triton-X100 (Sigma-Aldrich #T8787) in PBS and non-specific antibody binding is blocked using a 10% solution of HiHS (Gibco #26050) in PBS for 1 hour. The DeadEnd TUNEL Labelling kit (ProMega #G3250) and the Click-iT EdU Alexa Fluor 647 Imaging Kit (Invitrogen #C10340) are used for visualizing apoptosing cells and

EdU incorporation respectively, according to manufacturers instructions. Glial cell proteins are visualised using primary antibodies raised against MBP (Serotec, 1:300), NG2 (Milipore, 1:200), O4 (Immunosolv, 1:1000), CD68 (Serotec, 1:200), GFAP (DakoCytomation, 1:500) and Olig2 (Millipore, 1:200). Cells are incubated with primary antibody diluted in blocking solution for either 1 hour at room temperature or overnight at 4°C. Cells are then incubated with the appropriate AlexaFluor secondary antibodies (Invitrogen) at room temperature for 1 hour and stained with Hoechst (1:2000) for 5 min. Coverslips are attached to all slides using Fluoromount G (Southern Biotech #0100-01).

2.4 nTreg isolation and culture

Brachial, axillary and inguinal lymph nodes as well as spleens are removed from Foxp3-GFP mice of at least 6 weeks of age. The tissue is mechanically dissociated using fine gauze and cells are suspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco #31870). Cells are then incubated with CD4 L3T4 Microbeads (Miltenyi #130-049-201) and the $CD4^+$ population is separated using an AutoMACS Pro machine (Miltenyi). These cells are suspended in RPMI 1640 (Gibco #31870) supplemented with 20% heat-inactivated fetal calf serum (HiFCS) (Sigma #F7524) and sorted for GFP expression using a FACSAria II cell sorter (BD Biosciences). $CD4^+$ GFP^+ and $CD4^+$ GFP^- populations are recovered from this protocol. The GFP^+ fraction is plated at $2x10^5$ - $5x10^5$ cells per well in 24-well plates (Costar #3524) coated with 2ug/ml of Anti-Mouse CD3e antibody (eBioscience #14-0031-86) and 2ug/ml Anti-Mouse CD28 antibodies (eBioscience #14-0281-85) (αCD3/αCD28). The plating medium is either RPMI supplemented with 10% HiFCS (Sigma

#F7524), 2mM L-glutamine (Gibco # 25030-024), 100U/ml penicillin (Gibco # 15140-114), 100ugml^{-1} streptomycin (Gibco # 15140-114) and 50uM 2ME (Sigma # M7154) or AIM V medium (a serum-free medium) (Gibco #12055) supplemented with 2mM L-glutamine (Gibco # 25030-024), 100U/ml penicillin (Gibco # 15140-114) and 100ugml^{-1} streptomycin (Gibco # 15140-114). Cells are incubated in 5% CO_2 at 37°C for 48 hours before the medium is removed and stored at -20°C between harvest and use.

2.5 iTreg generation

$CD4^+$ GFP^- cells recovered from the cell sorter are plated at $2x10^5$-$5x10^5$ cells per well on αCD3/αCD28-coated 24-well plates (Costar #3524) in RPMI 1640 (Gibco #31870) supplemented with 10% HiFCS (Sigma #F7524), 5ng/ml human recombinant TGFβ (RnD systems #240-B-002) and 100U/ml IL-2 as described previously [118]. After 5 days in 5% CO_2 at 37°C cells are re-sorted using the FACSAria II cell sorter (BD Biosciences) for GFP induction. Cells expressing GFP are plated at $2x10^5$ - $5x10^5$ cells per well on αCD3/αCD28-coated 24-well plates (Costar #3524). The plating medium is either RPMI supplemented with 10% HiFCS (Sigma #F7524), 2mM L-glutamine (Gibco # 25030-024), 100U/ml penicillin (Gibco # 15140-114), 100ugml^{-1} streptomycin (Gibco # 15140-114) and 50uM 2ME (Sigma # M7154) or AIM V medium (a serum-free medium) (Gibco #12055) supplemented with 2mM L-glutamine (Gibco # 25030-024), 100U/ml penicillin (Gibco # 15140-114) and 100ugml^{-1} streptomycin (Gibco # 15140-114). Cells are incubated in 5% CO_2 at 37°C for 48 hours before the medium is removed for analysis.

2.6 Summary of Media used for experiments

Complete Medium: RPMI supplemented with 10% HiFCS (Sigma #F7524), 2mM L-glutamine (Gibco # 25030-024), 100U/ml penicillin (Gibco # 15140-114), 100ugml^{-1} streptomycin (Gibco # 15140-114) and 50uM 2-mercaptoethanol (Sigma # M7154).

AIM V medium: (a serum-free medium) (Gibco #12055) supplemented with 2mM L-glutamine (Gibco # 25030-024), 100U/ml penicillin (Gibco # 15140-114) and 100ugml^{-1} streptomycin (Gibco # 15140-114).

X-vivo: X vivo 15 chemically defined serum free haematopoetic cell medium (Lonza), 2mM L-glutamine (Gibco # 25030-024), 50uM 2-mercaptoethanol (Sigma # M7154) 100U/ml penicillin (Gibco # 15140-114), and 100ugml^{-1} streptomycin (Gibco # 15140-114).

SATO medium (for OPCs): Dulbecco's Modified Eagle's Medium (DMEM) Sigma D-6546 containing 5000 U/ml Penicillin/Streptomycin solution, 4mM L-Glutamine Sigma G-6392, 16 μg/ml Putrescine Sigma P-5780, 400 ng/ml L-Thyroxine (T4) Sigma T-1775, 400 ng/ml Tri-iodothyroxine (T3), Sigma T-6397, 60ng/ml Progesterone, Sigma P-8783, 5 ng/ml Sodium Selenite, Sigma S-5261, 100 μg/ml Bovine Serum Albumin fraction V, Sigma A-4919, 5 μg/ml Insulin, Sigma I-1882 and 50 μg/ml Holo-Transferrin (human), Sigma T-0665.

Slice culture medium: 50% MEM with Earle's salts, 25% Earle's Balanced Salt Solution, 25% heat inactivated horse serum (HIHS), glutamax-II supplement with penicillin–streptomycin, amphotericin B (all purchased from Invitrogen) and 6.5 mg/ml glucose (Sigma).

2.7 Cytokine quantification

The quantification of cytokines was performed using a combination of FlowCytomix simplex detection kits specific for: mouse IL-10 (eBioscience #BMS8614/2FF), mouse GM-CSF (eBioscience #BMS8612FF), mouse IFN-gamma (eBioscience #BMS8606/2FF), mouse IL-17A (eBioscience #BMS86001FF), mouse IL-1alpha (eBioscience #BMS8611FF), mouse IL-2 (eBioscience #BMS8601FF), mouse TNF-alpha (eBioscience #BMS8607/2FF), mouse IL-23 (eBioscience #BMS86017FF), mouse IL-4 (eBioscience #BMS8613FF) and mouse IL-22 (eBioscience #BMS86016FF). All kits were used according to the manufacturer's instructions. A BD FACSCalibur Flow Cytometer (Becton Dickinson) was used for detection and the results were analysed with FloCytomix Pro2.4 software (eBioscience).

2.8 Stereotactic Surgery

Using anaesthetized 12 week old C57Bl/6 male mice, 2µl of 1% Lysophosphatidyl choline/Lysolecithin (LPC) was injected through a hole drilled in the skull at stereotactic coordinates 1.2mm posterior, 0.5mm lateral, 1.4mm deep to the bregma over 4 minutes using a 30 gauge needle attached to a Hamilton syringe, driven by a KD Scientific Nano pump, which was left in situ for 4 minutes to reduce backflow. Injection of T cells or nanoparticles were carried out not exceeding the same volume, using the same coordinates. At various time-points, mice were perfused with 4% PFA, and fixed brains were bisected coronally at the injection site.

2.9 Tissue immunohistochemistry/fluorescence

For staining of tissue sections, fixed tissue was cryoprotected in sucrose, frozen in OCT cryostat embedding medium (Tissue Tek, Agar Scientific) and 10µm sections were cut on a cryostat (Leica CM3050 S) and mounted

on superfrost slides (VWR International). Sections were washed in PBS and blocked in 10% normal horse serum in PBS containing 0.2% triton for 1 hour in a wet chamber at room temperature (RT). They were then incubated with primary antibody at 4°C overnight. The next day, the sections were washed in PBS, incubated with appropriate fluorescently labelled secondary antibody (AlexaFluor, Invitrogen) in a wet chamber for 2 hours at RT and washed in PBS again. Finally, the slides were mounted in fluoromount.

To detect demyelination, Luxol fast blue (LFB) staining was performed using a standard protocol. In brief, slides were left in LFB solution overnight at 60C. Put slides into LFB solution. Lithium carbonate solution, and then 70% ethanol was used to differentiate the colours, and cresyl fast violet was used as a counter stain.

2.10 Nanoparticles

Nanoparticles were obtained from Dr Su Metcalfe at the University of Cambridge, and manufactured by the Tarek Fahmy Laboratory in the Yale School of Engineering and Applied Science. These were made from PGLA and either contained LIF, or not (as a negative control). These nanoparticles have avidin on their surface and were incubated with biotinylated NG2 antibody (species rabbit) (Millipore) to allow targeting of OPCs. Nanoparticles were identified by using a fluorescent anti-rabbit secondary antibody.

Chapter 3: Results

The first step in this project was to investigate the Treg and their secreted cytokines in vitro and validate the methods that we intended to use for their culture and analysis. In particular it was important to validate the novel set-up of our in vitro Treg cultures and analyse the effect that this might have on the Treg themselves.

3.1 Regulatory T cell experiments

3.1 *Treg culture: survival and phenotypic stability*

Introduction

I decided to begin by investigating the effects of Treg-secreted cytokines on OPCs. Rather than attempting to co-culture Treg and OPCs, I initially focused on generating Treg-conditioned media to add to OPC cultures. Previous studies into the effect of T lymphocyte cytokines on oligodendrocytes have generally used single recombinant cytokines, such as interleukin-2, to investigate this relationship. I pursued the strategy of testing the 'whole' Treg supernatant, including all of the secreted cytokines, in representative relative amounts. I felt that this approach would more faithfully replicate the in vivo situation, where the reality is that T lymphocytes do not secrete cytokines singly (see Table 1). The initial step in this strategy was to establish a technique for culturing Treg without high levels of serum, which would boost the viability of OPCs when Treg supernatants were added to OPC culture and skew the results. This involved using serum-free media for Treg culture and two different media were tried and compared to "complete" medium: AIM V

media (Gibco) and X-vivo media (Lonza). Due to the novel culture set-up required for generating Treg cytokines, the stability and survival of Treg of both types in culture was assessed.

Method

The foxp3-GFP mouse first used by Fontenot et al. (2005) [165] was used as a source of both natural and induced T regulatory cells. Harvest and generation of Treg (see methods) involves the removal of spleen and lymph nodes from 6-10 week-old mice before the tissue is processed to remove blood cells and separate immune cells. Those T lymphocytes that express CD4 can then be separated using magnetic beads coated with anti-CD4 antibodies using the magnetic-activated cell sorting (MACS) system. This population of CD4+ T cells can finally be sorted, using fluorescence-activated cell sorting (FACS), for those expressing GFP (natural Treg) and for the binding of fluorescent antibodies to CD62L and CD25 (naïve T cells). Plating nTreg for 48h in varying media and in the presence of TCR and co-stimulatory antibodies (anti-CD3, anti-CD28) gives nTreg supernatant. Naïve T cells, plated in anti-CD3 and anti-CD28 coated wells with media supplemented with IL-2 (200U/mL) and TGFb (5ug/mL) for 5 days, gain expression of foxp3 and can therefore be sorted using FACS for GFP expression. A further 48h of cell culture gives induced Treg (iTreg) supernatant. Wherever possible, the use of the cytokine IL-2 was avoided as IL-2 has been reported to influence the proliferation and maturation [3]. I compared standard T cell culture medium (Complete medium) with AIM V media (Gibco) and X-vivo media (Lonza).

Results

nTreg

To obtain nTreg, the foxp3-GFP mouse where all cells that express foxp3 fluoresce green, and by FACS for GFP, I could obtain 100% GFP+ expression in plated cells (nTreg). After 48 hours in culture without IL-2, which is important for nTreg survival, the cell medium was removed and the cells analysed using a combination of FACS, to look for both their GFP expression and survival, and Trypan Blue (TB) staining, for the integrity of the plasma membrane. In the analysed cells, the proportion of nTreg expressing GFP typically dropped to 89.8% of total cells in complete medium and 99.93% and 90.1% in AIM V and X-vivo medium respectively (Fig. 4A), suggesting reasonable stability of the cell phenotype. However, cell survival was quite low as shown both by staining with TB and by the proportion of events in the 'live gate' of FACS analysis. When analysed by flow cytometry, the survival rates were 15.6% of total detected events in complete medium, 13.7% in AIM V and 16.35% in X-vivo medium (Fig. 4B). This trend was mirrored closely when analysed through staining with TB, counting approximately 400 cells per group. In complete medium 27.83% of cells survived, in AIM V the rate was 1.5% and with X-vivo medium, 19.32% (Fig. 4C). These very low survival rates can partially explain the very high rates of GFP expression observed as only a small proportion of very healthy cells, with robust GFP expression, remained after 48h without IL-2.

iTreg

With iTreg, a slightly different pattern was observed. Again, the GFP+ iTreg were sorted using FACS and only GFP+ cells were plated out, giving a culture with 100% GFP positivity. When GFP expression after culture for 48h in the absence of IL-2 is analysed, the frequencies of GFP+ cells are generally lower: 43.03% in complete medium, 80.92% in AIM V

medium (56.7% when supplemented with 50uM of 2-mercaptoethanol (2-ME)), and 51.12% when the cells were cultured in X-vivo medium.

As a preliminary experiment testing whether Treg have a stable phenotype in culture media optimised for oligodendrocyte culture, Treg were plated for 48h in the media from two such cultures. The eventual aim of this experiment was to see if co-culturing oligodendrocytes, either in vitro or ex vivo, with Treg was viable. Tests to analyse iTreg survival in the two oligodendrocyte medium solutions, Slice culture medium and SATO medium, resulted in GFP-positivity rates of 44.85% and 16.85% respectively (Fig.5A). Thus, stability of iTregs under all of these culture conditions was much lower than in previously tested culture media (complete media).

Survival of these cells was analysed in the same way as for the nTreg cultures, by FACS sorting and numbers of cells in the live gate and by trypan blue staining and microscopy. When using flow cytometry as a measure of cell survival, 32.88% of cells survived in complete medium, only 7.5% in AIM V medium, rising to 75.15% in AIM V medium supplemented with 50uM 2-ME and 64.7% in cells cultured in X-vivo medium. For slice culture medium and SATO medium only a very few cells survived: 0.30% and 24.4% respectively (Fig. 5B). In close agreement with this, the percentage of iTreg that were negative for TB staining was 49%, 6.73%, 82.34% and 71.29% respectively - clearly showing that iTreg survive well in AIM V supplemented with 2-ME and X-vivo medium (Fig. 5C). The frequency of cells surviving, as measured by staining for TB from slice culture medium and SATO medium was 1.17% and 28.85% respectively, which was relatively poor (Fig. 6). Thus, iTreg seem to survive better than nTreg under these culture conditions, but they are more likely to lose their marker of Treg status – Foxp3:GFP.

Summary

There is a loss of survival of Treg cultured in media without serum, which is more marked with nTreg than iTreg. However, the stability of Treg in culture in media without serum after 48 hours, as determined by their foxp3-driven GFP expression is also variable, but most stable in the AIM V medium with additional 2-ME.

Overall, the percentage of live cells expressing GFP was higher in cell populations suffering from poor survival. This is probably because only the 'healthiest' cells, which are assumed to have the strongest protein expression, including that of GFP, survive in these conditions. In essence, while 100% of a small surviving cell population might be GFP+, possibly only 50% of cells in a much larger surviving population may express GFP, meaning the absolute number of GFP+ cells is far higher.

I used these Treg supernatants to treat oligodendrocytes, as I expected that activated Treg would have secreted appreciable amounts of cytokines before losing GFP expression or cell death. This was preferred over the addition of relatively large amounts of IL-2 to the culture medium since this cytokine is known to boost oligodendrocyte proliferation [3].

3.2 *In vitro Treg culture supernatants contain cytokines*

Introduction

Having established a culture system for obtaining nTreg and iTreg supernatants in a variety of media, I analysed the supernatants in order to establish the types and amounts of cytokines that they contained. Since the Treg were cultured in wells coated with CD3- and CD28-stimulating antibodies, they were assumed to be in an activated state and therefore

likely to be secreting cytokines involved in their suppressive function, such as IL-10 and TGFb. We expected the Treg to be secreting these and other cytokines plus small amounts of growth factors that have been reported previously (see table 1). Establishing every cytokine that the Treg secreted was not my main objective, as it would be too technically demanding and time-consuming, rather my aim was to ask whether some of the cytokines that have previously been studied, such as IFNg and TNFa, were present in our supernatants. This would allow us to try and predict the effect that these supernatants would have on oligodendrocytes based on previous reports (see table 1).

Method

As there was no feasible way to detect all of the proteins secreted by Treg within the scope of this project, I instead looked for likely candidates using a customised 'flowcytomix' assay. This assay consists of a mixture of different fluorescent bead-bound cytokine-specific antibodies, which bind to the cytokine of interest and can be distinguished by the size of the bead and its fluorescent label using FACS. This provides a simultaneous analysis of ten or more cytokines from the same sample. I customised the kit that I used to include 10 cytokines of particular interest and tested for TGFb separately. The cytokines were selected following a literature search for cytokines that Treg were likely to secrete (as shown in table 1). TGFb must be assayed separately because it is secreted in an inactive form that is 'activated' under acidic conditions. Therefore in order to detect TGFb using an antibody against active TGFb the Treg supernatant must be acidified with hydrochloric acid, done using an eBioscience flowcytomix Simplex kit.

Results

Treg were extracted and cultured as previously described (see section 3.1). The supernatants from nTreg and iTreg cultured for 48 hours were removed and analysed for their cytokine content.

When cultured for 48h in complete medium, nTreg secreted few, if any, cytokines from my array, which included the archetypal Treg-secreted cytokine IL-10. The only cytokine detected consistently was IL-1a, and the level of this cytokine was close to the limit of sensitivity of the kit used and therefore may represent a false positive result (Fig. 7).

On the other hand, iTreg secreted a number of cytokines and in great amounts. Interferon gamma (IFNγ) was particularly highly expressed by these cells with almost 26ng/mL detected. The cytokines GM-CSF (0.153ng/mL), TNFα (0.127ng/mL) and IL-4 (0.146ng/mL) were also found in relatively large amounts in iTreg supernatants (Fig. 7). These results indicate that nTreg were not able to secrete cytokines well in culture, or that they express and secrete proteins that we did not test for. The absence of IL-10 is surprising, however it may be that poor survival of nTreg in these cultures reduced the level of secreted IL-10 to below the detection limit of these tests.

Testing for TGFb, relies on the acidification of samples, and therefore TGFb was assayed separately from other cytokines. Tests on Treg supernatants revealed a concentration of 671.60 pg/mL in iTreg supernatants, and 539.57 pg/mL in nTreg supernatants containing 10% fetal calf serum by volume (Fig. 8A,B). No TGFb was found in supernatants from cultures grown in serum-free media. Tests of the fetal calf serum used to supplement the media showed that the observed TGFb concentration could be entirely be accounted for by the serum content of the supernatants, as pure fetal calf serum contained a concentration of 5405.07 pg/mL TGFb (Fig. 8C). This constrains the levels of TGFb actually secreted by these cells to very low levels of free TGFb. A possible

explanation is that the TGFb produced by these cells is in the form of membrane-bound TGFb (mTGFB) rather than secreted, which would then require direct contact with oligodendrocytes or cleavage and release to have any biological effect in vivo.

Summary

These results therefore show that iTreg secrete a range of cytokines (IFNg, TNFa, GM-CSF and IL-4) but my cultured nTreg did not convincingly produce any of the cytokines I chose to measure, including IL-10. This is unexpected and may be related to the poor survival or lack of continued activation of nTreg in my culture conditions. Other cytokines or growth factors may also have been present, and it would have been interesting to measure these by QPCR.

I next aimed to determine whether these secreted factors had any effect on OPC biology.

3.3 *Treg supernatants have no effect on OPC proliferation in vitro*

Introduction

Having set up Treg cultures and harvested media from these cultures that contained a number of cytokines proven to affect oligodendrocytes, I began to test these supernatants on OPCs in culture. Our rationale was to find out if the combination of these different Treg-secreted cytokines would affect oligodendroglial cell behaviour particularly OPC proliferation, viability and differentiation which impact on the efficiency of remyelination in vivo. In response to CNS inflammation, proliferation is desirable to maximise the number of OPCs available for repair, increased viability should preserve the local population of OPCs available

for repair and enhanced differentiation into myelinating oligodendrocytes should provide the largest possible population of mature oligodendrocytes capable of remyelination. I began by testing the effect of Treg-conditioned media on proliferation, a reliable and easily analysed cell behaviour that is desirable for tissue repair.

Method

OPCs were obtained by the shake-off method from P0-P2 mice and plated on poly-D-lysine-coated 8-well chamber slides, with 20,000 cells per well. OPCs were cultured in SATO medium for 24h with PDGF and FGF at 10ng/mL b/v. After 24h, the medium was changed to 2X concentrated SATO medium diluted to 1X concentration with Treg supernatant and containing 5mM 5-ethynyl-2'-deoxyuridine (EdU), so that Treg supernatant accounted for 50% of the volume, but the growth factors and supplements in SATO were at the normal concentration. After 24h these cells were fixed and EdU incorporation in OPCs exposed to Treg supernatant was compared with incorporation in those that had been treated with vehicle T cell medium only.

Results

As mouse OPC cultures are less pure than rat cultures using the shake-off technique, I first measured the proportion of total cells that expressed Olig2, a classic oligodendrocyte marker, which was on average ~60%. Therefore, I needed to co-stain cells with a proliferation marker and an oligodendroglial marker to ensure that I was measuring the response of OPCs in the cultures to the Treg supernatants, and not other cells. Initially I used BrdU to identify proliferating oligodendrocytes but this marker did not prove to be reliable enough as surface antigens required for correctly identifying OPCs were destroyed by the required antigen retrieval step for BrdU immunostaining. However, the use of EdU

incorporation into dividing cells over time and an antibody to proliferating cell nuclear antigen (PCNA), labelling cells actively dividing at one time-point ultimately proved to be more consistent. 5mM EdU was added to OPC cultures 21 hours after the addition of Treg supernatants, 3 hours prior to fixation. At the same time, fixed cultures of OPCs were also immunostained for PCNA.

In OPCs treated with supernatants from nTreg, the level of EdU incorporation of oligodendroglial cells was almost identical to control levels (Fig. 9). This was the case regardless of whether nTreg had been cultured in complete medium (Fig. 9A), AIM V medium (Fig. 9B) or X-vivo medium (Fig. 9C). PCNA staining followed a similar pattern and did not vary significantly from control levels with any treatment.

The same was true of OPCs treated with supernatants from iTreg cultures. Again, in these cultures approximately 60% of cells expressed the oligodendroglial marker Olig2. There was no difference in EdU incorporation with OPCs treated with iTreg supernatant using complete medium (Fig. 10A), AIM V medium (Fig. 10B) or X-vivo medium (Fig. 10C). PCNA staining followed a similar pattern and did not vary significantly from control levels.

Summary

Therefore, neither nTreg nor iTreg supernatants had any significant effect on oligodendrocyte proliferation. The OPC proliferation rate did vary depending on the type of culture media added to the OPCs - EdU incorporation when X-vivo medium was added was lower generally, at approximately 40% of cells compared to approximately 60% of cells with the other culture conditions (Fig. 9C, 10C). However, there was no difference between proliferation rates in OPCs treated with Treg

supernatants compared to those that had not been conditioned by Treg using any type of media.

3.4 *Treg supernatants have no effect on OPC differentiation in vitro*

Introduction

The second cellular behaviour that I analysed in the presence of Treg-secreted cytokines was the differentiation of oligodendrocytes. Within the scope of these experiments 'differentiation' was defined as changes in OPC biology which led to surface expression of NG2 followed by O4 and then, most importantly, MBP. Boosting the number of MBP-expressing oligodendrocytes within and around demyelinated lesions may make successful remyelination more likely. Some reports describe a 'differentiation block' amongst OPCs in lesions where many OPCs are found but very few differentiated oligodendrocytes [79]. This makes finding ways of boosting OPC differentiation all the more important.

Method

OPCs were obtained via the shake-off method and plated at a density of 20,000 cells per well into 8-well chamber slides containing SATO-medium supplemented with 10ng/mL PDGF and FGF. After 24h the medium was removed and 2X SATO medium diluted to 1X SATO with Treg supernatant was added to some wells and 2X SATO medium diluted with 'vehicle' medium added to others as a control. 24 and 72 hours later, the cells were fixed and the cells immunostained for MBP and Olig2. Cells were imaged using a confocal microscope, blind-counted and the number of MBP-expressing cells was calculated as a percentage of total Olig2+ cells.

Results

Initially three different oligodendrocyte markers were used to try and determine the extent of differentiation of OPCs. The OPC-specific proteins chondroitin sulphate proteoglycan (NG2), sulphatide (O4) and myelin basic protein (MBP) were used to identify OPCs in early, intermediate and mature stages of differentiation (Fig. 2). Unfortunately this technique relied on all markers having optimal immunostaining and this was not always the case. This method had been developed by a colleague in the MS centre using rat OPCs, but was less reliable in mouse OPCs, possibly as the O4 antibody used was of mouse origin. Therefore, I elected to use only the very reliable MBP immunostaining to quantify OPC maturation. This is arguably more relevant to the assessment of the remyelinating potential of these cells, as studies of human lesions show that some OPCs appear to suffer so-called 'differentiation block' and fail to mature into MBP-positive oligodendrocytes [79].

When supernatants from nTreg were added to OPCs as above, there was no significant increase in the number of MBP-positive cells regardless of whether the Treg were originally cultured in complete medium (Fig.11A), AIM V medium (Fig.11B) or X-vivo medium (Fig. 11C). In all cases the percentage of all cells that expressed MBP was between 0 and 5%. As this appeared to be rather variable, I investigated whether this varied with the litter of mice used. I plotted the amount of MBP expression of OPCs according to which litter of mice that they came from, rather than the treatment they received, and there appeared to be less variation using OPCs within litters, and bigger differences in the OPC maturation between litters than between treatment groups (Fig. 11D). This suggests that the precise age of the pups used to extract OPCs, the environment of the mother or genetic factors may have more of an effect on MBP

expression in these cells than the effect of Treg supernatants. This was true even using 2 different litters of mice on the same day.

With iTreg supernatants, a similar set of results was observed. Again, between 0 and 4% of total cells in these OPC-enriched cultures expressed MBP and this did not vary significantly from control cultures in any of the three types of media; complete medium (Fig. 12A), AIM V supplemented with 2-ME (Fig. 12B) or X-vivo medium (Fig. 12C). Similarly to nTreg, there seemed to be more of a correlation between the litters that OPCs had been harvested from and MBP expression rather than between MBP expression and supernatant type (Fig. 12D).

Summary

Therefore, Treg supernatants have no effect on OPC maturation in vitro.

The number of MBP positive cells in these cultures generally was low in all of these experiments, which makes interpretation of differences difficult. It is apparent that mouse OPCs mature slower than rat OPCs in culture, and therefore it may be worth repeating this at a later stage in culture, when more cells have matured in the control group.

3.5 *Treg supernatants have no effect on OPC apoptosis in vitro*

Introduction

During CNS demyelination, regardless of whether it is initiated by CNS inflammation or demyelinating substances such as cuprizone or LPC, involves the death of oligodendrocytes. Protecting oligoendroglial cells from apoptosis by providing trophic factors that boost their heath, rather than by blocking apoptotic signalling, may be of therapeutic benefit. I

hypothesised that protecting pre-existing OPCs from cell death in the environs of a demyelinating lesion would boost the population of OPCs available for repair and therefore be beneficial to subsequent remyelination. To analyse apoptosis in vitro in response to Treg-secreted cytokines I added Treg supernatants to OPCs and quantified the number of OPCs undergoing apoptosis as measured by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL).

Method

OPCs obtained using the shake-off method were plated at 20,000 cells per well in 8-well chamber slides. After being exposed to the growth factors FGF and PDGF at 10ng/mL concentration the media was withdrawn and replaced with either 2X SATO media diluted to 1X with various Treg supernatants or 2X SATO medium diluted to 1X with vehicle medium. OPCs were fixed at 24 or 72h and apoptotic cells were visualised using a TUNEL assay. OPCs were also immunostained with anti-Olig2 antibodies to establish the number of apoptotic OPCs as a percentage of total OPCs at each timepoint.

Results

To analyse the effect of Treg-conditioned media on OPC cell survival in vitro, OPCs were harvested from the cortices of P0-P2 mouse pups and cultured for 24h, at which point Treg supernatants were added. These cultures were maintained for either a further 24 or 72h before being fixed and apoptotic cells were visualised with terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL). These time-points were chosen to look for the effect of Treg supernatant on immature OPC and more mature oligodendroglial cells. By co-staining for Olig2 it was possible to assess the number of oligodendroglial cells undergoing

apoptosis. Due to the level of double-labelling being very low the ratio of the total number of apoptotic bodies to cell nuclei was also measured to give an 'apoptotic index'. On both of these measures there was no significant difference between control and Treg-conditioned media (Fig 7).

As with previous cultures, the percentage of total cells in these cultures that expressed Olig2 was between 60 and 70% and this did not vary between treatments. At 24h after the addition of nTreg supernatants from nTreg grown in complete medium, there was no difference in apoptosis between both control and nTreg-treated media (Fig. 13A). At 72h, there was more apoptosis, but again with no significant difference between control and conditioned media (Fig. 13B). When apoptosis was measured as a percentage of Olig2+ cells that were also TUNEL+ the proportion of cells undergoing cell death was very low, not even reaching more than 1% in either conditioned media or control at 24 (Fig. 13C) and 72h (Fig. 13D). The suggestion from these experiments is that the components of nTreg-conditioned complete medium have no effect on OPC cell survival. These experiments were repeated with nTreg-conditioned X-vivo medium instead of complete medium, as a serum-free alternative to the complete medium assayed previously. Measurements of 'apoptotic index' and of the co-staining of Olig2 and TUNEL showed an apparent trend towards lower rates of apoptosis in the Treg-conditioned media (Fig. 14), but this is not statistically significant.

At the same time the effect of similar media conditioned by iTreg instead of nTreg was assayed. Using the same culture set-up and analysis, the effect of iTreg-conditioned X-vivo media on OPC cell survival was measured. When the apoptotic index was calculated at 24h (Fig. 15A) and at 72h (Fig. 15B) there was no significant change from the control values,

though again it was rather variable. When assessing death as a percentage of the total Olig2+ population that also stain with TUNEL there was, again, no significant difference between conditions at 24 (Fig. 15C) or 72h (Fig. 15D), but with much variability.

Summary

Therefore, Treg supernatants have no effect on oligodendroglial survival in vitro. The variability that I have seen in these experiments may be due to the very low level of apoptosis in these cultures, and this could be repeated using a deprivation medium (lacking serum and growth factors) to stimulate a higher death rate and to determine whether the Treg supernatant addition can rescue this. Alternatively, this variability may be related to the variation in cytokine amounts released by Treg in different cultures on different days, in spite of having done the experiment in the same way each time.

Summary of Results so far

I found no difference in OPC proliferation, maturation or death by applying Treg supernatants to OPC cultures. There were some problems with these experiments, in that the survival of Treg in culture, when the supernatants were being formed, was sometimes low, and the stability of these cells in terms of remaining Treg (at least by Foxp3-GFP expression) was variable and certainly not 100%. This may have influenced the type or amount of cytokines produced into the supernatants at each extraction. This may account for some of the variability seen in each of these experiments, making it difficult to discern any effect. Secondarily, mouse OPCs are much harder to extract from brain successfully, mature slower and may be more variable between extractions than rat OPCs (the standard used in the MS centre), compounding matters. To circumvent some of the problems of Treg culture, stability and variability of

supernatants, I decided to perform pilot studies of demyelinated brain lesions in vivo, to determine whether it was feasible to add Treg directly to these lesions and determine any effect on remyelination in a more representative cellular environment.

3.6 *Chemically-demyelinated lesions contain no T lymphocytes*

Introduction

My hypothesis was that Treg, injected into a chemically demyelinated lesion, would enhance the spontaneous remyelination of such a lesion. My first aim was to characterise the immune cell types already found in demyelinated lesions in order to better understand how the microenvironment would affect the behaviour of injected Treg. I looked for the presence of T lymphocytes and antigen presenting cells, as it would be desirable for Treg to receive endogenous TCR and co-receptor stimulation through interactions with MHC class II molecules to increase their cytokine output.

Method

Stereotactic apparatus was used to inject 2uL of LPC into the corpus callosum of 10-week old mice under anaesthetic. Mice were then perfused intracardially with 4% paraformaldehyde and the brains post-fixed in 15% sucrose solution for 24h followed by 30% sucrose for a further 24h. Tissue was then embedded in OCT before being sectioned using a cryostat and stained using luxol fast blue to visualise lesioned areas. Adjacent sections containing lesions were then immunostained for immune cells of interest.

Results

Injecting the chemical lysophosphatidylcholine (LPC) into the corpus callosum leads focal demyelination. I assessed whether T lymphocytes, and especially Treg, infiltrate the site of demyelinated lesions in this model. Brain tissue taken from mice that received LPC injections 3- and 4-days previously was immunostained using the T cell marker CD4 and the Treg marker foxp3. It was also analysed for the presence of microglia (Fig. 16A) and astrocytes (Fig. 16B) to test for innate immune system activation. Splenic tissue was used as a positive control for antibody staining, and contained both CD4-positive (Fig. 16E) and foxp3-positive T cells (Fig. 16F), yet no such cells were found in this brain tissue (Fig. 16C,D). As one article had suggested a brief influx of T cells into similar lesions in the spinal cord at 8 hours after lesion [169], I assessed tissue 8 hours and 24 hours after LPC injection, but again no T cells were seen. This led me to conclude that T cells do not infiltrate corpus callosum lesions after LPC injection. The difference in findings between LPC injection in the spinal cord and the corpus callosum may be related to the fact that the spinal cord is much smaller and lesioning it may produce enough inflammation, relative to volume, to allow entry to T cells through more breakdown of the blood brain barrier locally.

Therefore, in our model, the adaptive immune response is not activated, perhaps making it ideal to see whether factors produced by Treg have an effect directly on remyelination with no chance of an effect on T cells in the lesion, as there are not any present. Hence, it is a clean and simple system to answer the specific question of whether factors from Treg directly influence OPCs and remyelination.

3.7 *Naïve T cells do not survive in LPC lesions*

Introduction

Next, I analysed how well T lymphocytes survive injection into mouse brain and whether they persist in vivo. This was to help determine how many Treg should be injected and whether this would need to be repeated and how often.

Method

CD45.1 mice were lesioned using stereotactic apparatus to inject 2uL of LPC into the corpus callosum. For reasons of numbers, I used naïve T cells which were obtained from CD45.2 mice using the FACS technique to sort out CD4+ CD62Lhi CD25- cells. These were injected into CD45.1 mice, with a focal demyelinated lesion, to allow me to use specific antibodies to ensure that any Tcells found in the lesion could be identified as host (CD45.1) or transplanted (CD45.2). 10,000 naïve T cells were injected into the lesion either 24h following LPC injection. This number was chosen as it has been calculated in the Anderton lab that there are approximately 10,000 Treg present in brains of mice recovering from EAE, and it is known that this number is sufficient for that recovery (data unpublished). Mice were perfused intracardially with 4% paraformaldehyde 24, 48 or 72h following T cell injection and were post-fixed with 15% sucrose solution for 24h followed by 30% sucrose for a further 24h. Tissue was embedded in OCT and sections were obtained using a cryostat. Cryostat sections were stained with luxol fast blue to indicate the location of demyelinated lesions and sections containing lesions were immunostained for the presence of immune cells with antibodies against CD4 (to visualise T cells) and CD45.2 (to visualise injected cells) (compared to CD45.1 endogenous cells).

Results

After injection of 10,000 naïve T cells from CD45.2 mice into the lesion site of CD45.1 mice 24h or 5 days after LPC treatment, no T cells were seen in or around the lesions by immunofluorescence staining.

One hypothesis for this absence of cells is that the injections of T cells in this initial experiment may have been too soon after the LPC injection; therefore enough toxin may have still been present to damage the T cells. Thus, we repeated the experiment 5 days post-LPC injection to inject T cells and checked for their presence after 24 hrs. However, again, no T cells were identified.

This absence of local T cells may be because the cells used were naïve, not activated before injection, and therefore would not be primed to respond to the demyelinated lesion environment. They may have been unable to respond to the inflammatory cues of the demyelinated lesion, and simply migrated back into the bloodstream. The absence of transplanted T cells in the lesion discouraged me from attempting to inject Treg into LPC-induced demyelinated lesions and suggests that in these lesions, T cells do not receive the correct molecular cues necessary to persist locally in previously demyelinated tissue.

At this stage of the project, I decided to switch to a more candidate-based approach, selecting a candidate known to be released by Treg and which may affect OPC behaviour and remyelination. Work done in the Anderton lab to determine whether the cytokine profile of Treg isolated from the CNS and from the peripheral circulation are similar or different revealed that CNS Treg highly express the cytokine LIF at a level of 7 x higher than peripheral blood Treg. For this reason, and as LIF has already been shown to have effects on OPC function, I decided to focus on this cytokine. As before, I examined the effect of LIF on OPC proliferation, maturation and survival in culture, as these are important steps that OPCs must achieve

for successful remyelination and also investigated a novel nanoparticle delivery method for getting it to OPCs in lesion sites.

3.2 Leukaemia inhibitory factor (LIF)

3.2.1 *The T cell secreted cytokine leukaemia inhibitory factor (LIF) does not significantly increase OPC proliferation*

Introduction

As LIF is highly expressed by CNS Treg (unpublished data) and has well documented beneficial effects on both CNS inflammation [145-148] and oligodendroglial cell behaviour [154-156,170] , I decided to test its efficacy in vitro in the same way that I tested Treg supernatants. I first tested whether OPCs grown in media containing recombinant LIF proliferated more than controls.

Method

As an initial experiment to test the effect of LIF on OPCs in vitro, OPCs were harvested from P0-P2 mouse pups using the shake-off method as before and cultured for 24h in SATO medium supplemented with FGF and PDGF at 10ng/mL concentration. This was then removed and replaced with SATO just containing murine LIF (mLIF). mLIF produced from COS7 cells (obtained from the centre cell culture facility) was tested at a range of concentrations from 50, 100, 200, 500 or 2000U/mL to attain a dose-response profile for its effect on OPC proliferation. As a positive control for OPC proliferation, the proliferative factor PDGF was added at 10mg/ml. As a negative control, cells were incubated just in SATO medium. After 21h the medium was supplemented with 5mM EdU and fixed using 4% paraformaldehyde 3h later. Fixed cells were assayed for EdU incorporation and immunostained for Olig2 to indicate the

percentage of OPCs that incorporated EdU. Cells were imaged using a confocal microscope and blind counted.

Results

In these cultures, as before, the proportion of cells which were oligodendroglial (expressing Olig2) was constant at around 60% of total cells and did not vary between conditions. Adding 50, 100, 200, 500 or 2000U/mL of mLIF to these OPC cultures had no significant effect on OPC proliferation as assessed by EdU incorporation over 3 hours, between 21 and 24h post-addition of mLIF and immediately prior to fixation (Fig. 17A). For two of the three experiments, the positive control (10ng/mL PDGF) produced a robust increase in proliferation, however in one experiment the positive control value showed anomalously low EdU incorporation despite other wells, including the normal control, appearing normal (Fig. 17A). It is not known what caused this result. The trend in these experiments is that LIF increases proliferation, but this is not statistically significant, using an ANOVA test. Very high concentrations of LIF appear toxic to OPCs. This would mirror what has been found by other research groups [156].

3.2.2 *The T cell secreted cytokine leukaemia inhibitory factor (LIF) does not affect microglial proliferation*

Introduction

Since injecting LIF into the site of demyelinated lesions would be expected to stimulate LIF signalling in various other cell types besides OPCs, the effect of LIF on microglia, found in large numbers at the site of demyelinated lesions, was investigated. In response to demyelination, microglia are activated and accumulate at the site of injury in large

numbers. Modifying the behaviour of responding microglia is the focus of others in the MS centre, but an indication of the microglial response to exogenous LIF would be useful for refining my hypotheses.

Method

Microglia were obtained as a by-product of the 'shake-off' method of obtaining OPCs. After separation microglia were placed in 8-well chamber slides at a density of 20,000/well and nourished with SATO medium supplemented with FGF and PDGF at 10ng/mL concentration. After 24h growth factors were withdrawn and LIF was added to wells at varying concentrations. After a further 21h of culture 5mM EdU was added and the microglia were fixed with 4% paraformaldehyde 3h later. After immunostaining for the microglial cell surface marker CD68, the nuclei were stained with Hoechst and cells were then imaged with a confocal microscope. The images were blind counted and the number of EdU-incorporating cells was expressed as a percentage of total CD68 cells counted.

Results

The proportion of microglial cells as a percentage of the total number of cells in culture was not affected by LIF concentrations of 50, 100, 200, 500 and 2000U/mL (Fig. 17C). This proportion remained at 75-80% regardless of treatment. Although the experiment was only performed twice, and so statistical tests cannot be used, there was no effect of LIF on microglial proliferation at any concentration (Fig. 17C).

3.2.3 *The T cell secreted cytokine leukaemia inhibitory factor (LIF) does not significantly increase OPC differentiation*

Introduction

I went on to apply the same technique to testing whether OPC maturation (as defined by MBP expression) is enhanced by the application of LIF in vitro. Boosting the number of MBP-expressing cells in the context of a demyelinated lesion would be expected to boost the chances of effective remyelination, a desirable therapeutic outcome. My expectation was that LIF would increase the number of MBP+ cells.

Method

OPCs were extracted from P0-P2 mice using the shake-off method as before. Harvested OPCs were then cultured in 8-well chamber slides at a density of 20,000 cells/well. These cells were cultured in SATO medium supplemented with FGF and PDGF at 10ng/mL concentration for 24h before the media were replaced with SATO containing mLIF at varying concentrations. After 72h the cells were fixed with 4% paraformaldehyde and immunostained for Olig2 and MBP. Imaging using a confocal microscope and blinded counting gave the percentage of Olig2 cells that co-expressed MBP at 72h.

Results

Differentiation, as measured by the percentage of cells which become MBP+ after 72h in culture is not significantly affected by the addition of LIF to cultures of mouse OPCs. mLIF does not change the proportion of oligodendroglial cells in these cultures at 72 hours remaining at around 60% Olig2+ cells regardless of the culture conditions. Although there is a trend increase towards greater numbers of MBP+ cells (typically 0.5-4% of the Olig2+ population) at greater concentrations of mLIF, it is not statistically significant (using ANOVA) (Fig. 17B). Again the variability of values in each condition was large and the low level of MBP

expression at 72h in mouse OPCs makes attaining a significant result from this experiment technically challenging.

3.2.4 *The T cell secreted cytokine leukaemia inhibitory factor (LIF) does not significantly affect OPC motility*

Introduction

Using a single recombinant cytokine for experiments rather than Treg culture supernatant allowed us to test the motility of OPCs. The volume of supernatant required for these Transwell experiments made this previously unfeasible. I also decided to use rat OPCs rather than the more challenging mouse OPCs to assay the effect on LIF on OPC movement. Rat OPCs were necessary because of the large numbers of cells required. The assay involves placing OPCs on a porous membrane in the upper chamber, and placing cell medium containing LIF in the lower chamber to set up a chemotactic gradient. The number of OPCs that have moved from one side of the membrane to the other at a timepoint is quantified, thus determining the motility of the OPCs.

Method

OPCs were harvested from the CNS tissue of rats by the 'shake-off' method. The OPCs, plated on the membranes of Transwell inserts at a density of 75,000 per well, were nourished with SATO media supplemented with increasing concentrations of LIF (500, 200 and 100U/mL). PDGF at a concentration of 10ng/mL was used as a positive control as it is known to increase motility and is chemoattractant to OPCs. Cells were left for 16h, a time long enough to determine the effects of LIF upon the motility of OPCs without equilibration of the LIF gradient. After 16h, the OPCs were fixed with 4% paraformaldehyde and

0.01% glutaraldehyde for 30 min and the nuclei were subsequently stained with Hoechst dye. Cells on the top of the membrane were cleaned away using a cotton bud and those on the underside of the membrane imaged and blind counted from a total of 5 images taken from each well.

Results

There was a strong trend towards increased motility in response to LIF at the lower end of the range of concentrations used but no statistically significant effect (using ANOVA) (Fig. 18). The positive control PDGF consistently increased motility and I predict that with more repetitions this trend towards increased motility with LIF may become significant.

Summary

Therefore, from my in vitro assays with LIF, I saw no effect on OPC proliferation or maturation, but a trend to an effect on migration. However, previous literature has also seen effects on proliferation and maturation. This persuaded me that delivery of LIF to OPCs, may be useful in vivo to aid remyelination.

3.2.5 *LIF-nanoparticle dynamics – pilot study*

Introduction

In collaboration with Dr. Su Metcalfe at the University of Cambridge we next sought to test the ability of biodegradable nanoparticles to deliver LIF to oligodendrocytes in vivo. Since Treg themselves do not appear to be useful for this application, a viable alternative may be to supply oligodendrocytes with Treg cytokines through this method. The first stage towards accomplishing this goal was to determine the dynamics of the nanoparticles in vivo, since I planned to use them to help repair

demyelinated lesions. I obtained these nanoparticles directly from Dr Metcalfe, who had manufactured them to contain LIF, (or no LIF as a control) and I chose to use nanoparticles coated with the biotinylated antibody NG2, to aim to target them to NG2+ OPCs.

Method

Three groups of 10-week-old mice were used. In the first group of 3 mice, I injected 0.01mg of nanoparticles coated with biotinylated anti-NG2 into the corpus callosum, and immediately sacrificed the mice, to ensure that nanoparticles could be detected. The second group of 3 mice were treated identically but perfused after 72h, to determine how long nanoparticles were detectable. The third group of 3 mice had demyelinated lesions made in the corpus callosum using a 2uL injection of the chemical LPC, and 5 days later I injected 0.01mg of nanoparticles coated with biotinylated anti-NG2 into the site of the lesion, and culled these 72h later. This group was to determine whether nanoparticles either accumulated more in such lesions or did not survive such lesions.

All mice were intracardially perfused under general anaesthetic with 4% paraformaldehyde and the brains were removed and post-fixed in 4% paraformaldehyde for 24h before being immersed in 15% sucrose solution and then 30% sucrose solution 24h later. Brains were then cut in half with a coronal section through the site of injection. The rostral and caudal sections of brain tissue were then embedded in OCT and 10um sections were made using a cryostat. All sections were stained using luxol fast blue, in the lesioned brain this was used to locate the lesion and in the other two groups simply to visualise the tissue structure and look for the site of nanoparticle injection. Nanoparticles were detected by using an anti-rabbit fluorescent secondary antibody, as the NG2 antibodies used to coat the particles were of rabbit species, and this method had been successful at detecting these in vitro.

Results

NG2 coated nanoparticles are difficult to detect after injection into the CNS. I located the site of injection in the first two groups of animals by looking for the needle track in sections but no obvious accumulation of nanoparticles was visible, even in the first group. In the third group the lesion site was clearly visible on luxol fast blue stains of the corpus callosum and yet no nanoparticles were visible. This was surprising since the nanoparticles should not have degraded over so short a time-period. It may be that the nanoparticles were ingested by microglia or macrophages very rapidly and subsequently broken down and this is a problem which has been noted previously in the nanoparticle literature [171]. The other possibility is that the nanoparticles quickly entered the blood and were cleared from the area of injection. To resolve this issue, I propose labelling the nanoparticles directly with fluorescein, to ensure easy detection.

Thus, although nanoparticles targeting OPCs are potentially a very interesting way to deliver factors locally, and affect OPC function, there are some technical difficulties to overcome.

Chapter 4: Discussion

Here I have presented the results of my project aiming to discover whether Treg-derived cytokines, en masse or individually, have beneficial effects on CNS remyelination. Although I have been unable to prove or disprove this hypothesis conclusively, I have reached several conclusions regarding the cytokines that different types of Treg secrete, the effect that these cytokines have on OPCs *in vitro* and the effect of one of these cytokines, LIF, has on OPCs *in vitro*.

4.1 *Regulatory T cell-secreted cytokines*

My aim was to establish the effects of 'whole' Treg secretions, rather than individual cytokines, on remyelination in the central nervous system, with the idea that the cytokines they produced would interact producing synergistic or opposing effects which would be impossible to determine by examining each cytokine separately.

One of my first objectives was to establish the composition of these secretions, by assaying Treg-conditioned media for known cytokines. I had several expectations prior to these assays. I hypothesised that Treg cultured *in vitro* would secrete IL-10 [172] and possibly TGFb due to previous work demonstrating the immunosuppressive functions of IL-10 [173] and TGFb [174]. In my assay, I detected a range of cytokines such as IFNg, GM-CSF, IL-4 and TNFa in supernatants from iTreg culture. This complement of cytokines agrees with other work from the Anderton lab that significant amounts of IFNg and GM-CSF are secreted. Very few cytokines were detected in supernatants from nTreg, contrary to expectations. The cells used in these experiments were activated *in vitro* via co-stimulation of the TCR and the co-receptor CD28 using activating

antibodies, and therefore would be expected to secrete cytokines, however, there may be some differences in this system compared to *in vivo*.

4.2 *Suitability of in vitro culture*

Is stimulation in vivo likely to be similar to T cell activation using TCR and CD28?

Since the environment of a demyelinated lesion is typically inflammatory, I expect that Treg found in this environment will be similarly activated by antigen binding to the TCR and co-stimulation by CD28.

Did contamination by other T cells in culture skew the cytokine results?

I do not believe that there was a significant contamination of the Treg cultures by other CD4+ T cells. During the sorting process the CD4+ T cells were extracted from whole spleen and lymph node extract using MACS. Of this CD4+ population, a well-defined nTreg sub-population expressed GFP. This provided a distinct target to sort using FACS without the sometimes subjective boundaries between high, medium and low expression that some populations of cells require. For iTreg too there was a distinct population of GFP+ cells, so it is possible to say with confidence that 100% of the plated cells expressed GFP and therefore also foxp3 – the cardinal marker of regulatory T cells.

Do Treg in culture alter their phenotype, hence changing cytokine profiles?

There is little or no evidence to suggest that Treg can de-differentiate or even 'trans-differentiate' into T helper cells under any conditions, so I do not expect that any activated T helper cells will have been generated *in vitro* following plating. Furthermore, I tested this hypothesis by isolating GFP+ Treg, and quantifying the percentage of cells that retained their GFP+ 48 hours, and only saw a minimal reduction in GFP+ cells.

Does my Treg cytokine assay fail to identify pertinent cytokines as it simply measures concentrations in large volumes of supernatant and may not reflect local high concentrations that would occur during close cell contact?

One important difference between our *in vitro* cultures and the *in vivo* situation is the size of the Treg population. From other work in the Anderton lab, the population of nTreg in the CNS of a mouse at the peak of EAE has been estimated at approximately $1x10^4$ cells. For the purposes of my experiment, the Treg were plated, wherever possible, at a density of $5x10^5$ per well. This is deliberately high in order to pool the maximum number of cells and get the most accurate picture of the secretions from these cells. *In vivo* the concentration of these cytokines would be expected to be much lower, but in a much smaller volume than the 2mL of medium used in these cultures. However, this large volume of medium was necessary for generating the large amounts of supernatant to add to oligodendrocytes *in vitro*. One 2mL well of Treg culture

typically provides enough supernatant for applying to only 7 oligodendrocyte wells.

Is there a difference in cytokine production between peripheral blood Treg and CNS-residing Treg?

The population of nTreg used in this study is from spleen and lymph nodes, and is not antigen specific, as opposed to the myelin-reactive CNS-based Treg that would be expected to reside in a demyelinated lesion. Unpublished work in the Anderton lab has identified that these two subsets of Treg show different gene expression patterns by microarray- hence my subsequent interest in LIF, which is expressed seven times higher in CNS Treg than peripheral Treg.

Does the media used to culture Treg influence their cytokine production?

Initially the two Treg subtypes were cultured exclusively in the optimised cell medium for Treg cultures, according to the Anderton lab. This includes a relatively high proportion of fetal calf serum (10% b.v.) and, to enhance Treg survival, 100 units of activity per ml of the cytokine IL-2. Both serum and IL-2 have an effect on oligodendrocyte cell processes, such as cell survival, proliferation, and differentiation [3], and therefore, using this medium, it would be difficult to identify any additional or antagonistic effect of Treg supernatant. For example, in one of my early experiments quantifying apoptosis in oligodendrocytes

treated either with a vehicle control (Treg medium only containing 10% serum and IL-2) or Treg-conditioned medium (also containing these components), apoptosis was very low and much lower than normal for oligodendrocytes in culture in both groups. Therefore, I attempted to culture Treg in serum-free medium, with or without IL-2. This allowed Treg media to be added to oligodendrocytes without a great boost in the level of serum affecting the results. Two serum-free media were used - AIM V, a T cell medium from Gibco, and X-vivo, from Lonza. It is possible that these affected the cytokines released by activated Treg in culture, and as survival was affected in these suboptimal media, this may also have influenced cytokine release and may account for some of the variability I subsequently saw when I applied the supernatants to oligodendroglial cultures.

4.3 *Effects of Treg cytokines on oligodendrocytes*

Having generated and analysed supernatants from several different Treg cell media I then moved on to testing their effects on oligodendrocytes. I tested three different processes in oligodendrocytes that I hypothesised would affect the outcome of remyelination; proliferation, differentiation and the rate of apoptosis. However, Treg supernatants showed no significant benefit to any cell process in oligodendroglial cells *in vitro*. However, I am not fully confident that this finding in my in vitro experiments is necessarily secure, due to the following difficulties:

Murine OPCs are much more vulnerable than Rat OPCs in culture

The T cells in this project were obtained exclusively from mice, due to the ubiquity of transgenic mice in immunology research and my need to use GFP to select out Treg. I then applied supernatants to mouse OPCs, but extracting OPCs from mouse tissue is more technically challenging than rat OPCs. One rat can yield as many OPCs as ten mice, and they are cultured more easily. The reasons for this are unclear, and the increased size of the rat brain cannot on its own explain the increase in numbers. The poor yield and fragile culture of murine OPCs obviously makes experiments with these cells proportionally more difficult than using rat OPCs, which is the norm in my laboratory. For example, the MBP expression of murine OPCs in culture is much reduced and occurs later than in rat OPCs. For mouse OPCs, at 72 hours, there are roughly 5% of cells stained with antibodies against MBP as opposed to around 20% for rat OPCs. This was a source of frustration throughout this project and led to an inevitable increase in the variability of such experiments. The low yields that come with murine OPCs also make some experiments, such as the transwell assay, requiring large numbers of OPCs, unworkable. With the move away from 'whole' Treg supernatants and towards LIF and LIF nanoparticles, the source of OPCs was changed from mice to rats. Subsequently the number and quality of OPCs was no longer an issue.

Staining to detect differences in differentiation was unreliable

Initially the objective with my differentiation experiments was to try and assess both the morphology and the immunomarkers of progressively differentiating oligodendrocytes. As oligodendrocytes progress from simple, bipolar cells through to highly-branched MBP-positive cells they successively express a number of cell surface markers; O4, NG2 and MBP. These markers overlap in their temporal expression somewhat. Initially I

attempted to classify the stage of each oligodendrocyte's differentiation based on whether they fell into one of five different categories; NG2+, NG2/O4+, O4+, O4/MBP+ or MBP+ only. I also intended to quantify how the morphology of an oligodendrocyte changes as it matures. Sorting cells into three categories; simple, complex and membranous, gives another indicator of the level of differentiation in an oligodendrocyte population. This technique has been used successfully by Huang & Jarjour et al. (2010) to quantify oligodendrocyte differentiation. While the immunocytological approach worked well occasionally, it was sensitive to poor or non-existent staining in a single channel, making such an experiment of little use. The number of experiments rendered ineffective by this encouraged me to simplify the staining protocol, settling on the reliable MBP staining as a marker of what one might refer to as 'terminally' differentiated oligodendrocytes, which are of the most relevance to successful remyelination. This approach was more reliable but no significant effect was observed with any of the Treg supernatants tested.

Quantification of apoptosis was problematic

For apoptosis, the reliable TUNEL assay was used successfully for every assay. Staining from TUNEL was bright, however the morphology of apoptotic oligodendrocytes made cell counting tricky. The process of apoptosis involves a number of stages where cells take on differing morphologies. In the first instance, the cell nucleus, and sometimes the cytoplasm, stains brightly. The cell then progressively 'blebs' into several smaller objects, which *in vivo* would be phagocytosed by macrophages, but *in vitro* simply degrade slowly over time. Fixation of culture at a pre-determined timepoints provides a snapshot of the cells apoptosing at that

point in time. The problem with counting arises when making a subjective decision on whether a group of TUNEL-positive blebs constitutes the remains of one or more cells. Most often this is obvious, as widely spaced cells in a field of view produce distinct groups of blebs. However, in dense groups of cells this becomes more difficult. During the process of apoptosis the nucleus is necessarily disrupted too, interfering with the localization of the normally reliable oligodendrocyte marker Olig2. To overcome these issues two counting methods were used. The first was a ratio of the total number of TUNEL-positive objects, including each of the blebs in an image, to the total number of nuclei. This removed the subjectivity of attempting to decide whether each group of blebs constituted one or more cells and also whether any perceived Olig2 staining was real. Instead this provides an objective measure of the 'level' of apoptosis in proportion to the overall cell density. This analysis assumes that the type of media and Treg cytokines present have no effect on the mechanism of apoptosis, for example increasing or decreasing the number of blebs produced per cell. The second counting method counted the number of objects co-staining for TUNEL and Olig2, with the assumption that these constitute the population of apoptosing oligodendroglial cells.

The Foxp3:GFP mouse has now been shown to have functionally defective Treg

One issue that arose during the course of this project concerned the transgenic mouse used to obtain the two types of Treg (natural and induced) that were used. The foxp3^{GFP} mouse, first generated by [165], expresses GFP fused to the amino terminus of the foxp3 protein that has been found to be essential for Treg development [114,175,176]. Recent work

studying the function of foxp3^{GFP} Treg in two mouse models of inflammatory autoimmune disease, the K/BxN model of inflammatory arthritis and the NOD model of diabetes, have shown that foxp3^{GFP} Treg are functionally deficient during inflammation. The fusion of the GFP protein to foxp3 appears to lead to steric hindrance that prevents the binding of proteins such as Hif1α [177] plus Tip60, HDAC7 and Eos [178]. This raises the question of whether Treg isolated from foxp3^{GFP} mice in this study can be assumed to be 'normal' Treg, with a representative cytokine secretion profile. Assuming that the mice used in our studies have no on-going inflammation, a reasonably safe assumption given their maintenance in a specific pathogen free (SPF) facility, their reliability depends on whether the pathways found to be affected are activated *in vitro*. The signalling pathways most relevant to the *in vitro* culture of iTreg are the IL-2, TGFb, CD28 and TCR pathways. The nTreg used in this study were not stimulated with anything other than CD3- and CD28-activating antibodies so only the TCR and CD28 are relevant to these cells. However, it may be that transgene has the altered physiological state of these Treg, which may in part explain the unexpected cytokine profile, and means that my findings of the effect of their supernatants on OPC behaviour in culture may not necessarily reflect *in vivo* activity.

T lymphocytes prefer different culture media to oligodendrocyte precursor cells

An early problem that I encountered in trying to add Treg supernatants to OPC cultures was the differing levels of serum content in the standard culture media for each cell type. T cells, and Treg, typically grow best in cell media containing 10% b.v. fetal calf serum, whereas mouse OPCs are best limited to only 0.5% - any higher and other glial cell types such as

astrocytes 'grow out' and take over these cultures. Boosting the serum content of OPC medium to 5% b.v. by adding Treg-conditioned media would be expected to also raise the rate of proliferation and reduce cell death to such an extent that detecting a small effect additional effect of cytokines in the media could be impossible. Attempts were made to culture Treg in serum-free media, firstly AIM V media (Gibco) and then finally X-vivo media (Lonza). Initially mixed results with AIM V medium led to the use of X-vivo medium, which worked far better. Eventually all three media were used in experiments to no effect with the conclusion that soluble Treg cytokines have no effect on OPCs *in vitro*. While this is suggestive that the same may be true *in vivo*, it could be argued that these experiments have little relevance to *in vivo* situations due to the problems described above and subsequent evidence as follows.

4.4 Are these results translatable to the in vivo situation?

In MS lesions there are few Treg present

Another consideration in applying the results detailed here to the in vivo disease situation in human MS patients is the relative abundance of OPCs and Treg within remyelinating MS lesions. It is known that OPCs occur in abundance in many MS lesions and that there is a need for factors that aid differentiation as these cells appear to suffer from a so-called 'differentiation block' [179]. A question that still needs to be answered is the abundance of Treg in these same lesions. Histological studies of MS biopsy tissue have shown that cells expressing foxp3 are present in early MS lesions[1]. However their density appears to be low, especially compared with the numbers of OPCs. The accepted model of Treg function in MS suggests that Treg act peripherally and it is not necessary for Treg cells to be present in the CNS. Considering the low levels of

cytokines secreted in vitro by activated Treg in this study and the low density of Treg shown in vivo, it seems unlikely that Treg-secreted cytokines alone could have any great effect on the maturity, survival and proliferation of OPCs, and thus remyelination. In our LPC model of chemical demyelination, we found no foxp3-expressing cells, in contrast to studies of human lesions[1]. As a result, it is unlikely that Treg and OPCs interact spontaneously in our model. We attempted to inject Treg into these lesions but we concluded that these T cells do not persist for long enough following injection in this model.

Early post-natal oligodendrocyte precursor cells may respond differently than adult OPCs

Some have suggested that remyelination is a recapitulation of development, with adult OPCs present in the adult CNS required to mature into myelinating oligodendrocytes in order to achieve remyelination. In this study, like many others, I used OPCs from the CNS of early postnatal mouse and rat pups rather than adult OPCs. These cells have a higher rate of proliferation [compared to adult] and typically mature in 3-7 days. It is likely that these cells may show different behaviours to OPCs present in the adult mammalian CNS. Thus, the fact that Treg do not appear to affect important cellular processes in these cells does not rule out the possibility that the same cytokines may affect the proliferation, maturation and survival of 'adult' OPCs.

Are in vitro cytokine concentrations representative of In vivo cytokine concentrations?

Not much is known about the in vivo physiological or pathological levels of the cytokines studied here. Serum measurements of various cytokine levels have been taken from MS patients (for example references see Kraus, J et al. (2002) [180] and Wang, KC et al. (2013) [181]). However, while the levels of these proteins at a systemic level may be useful, it does not show their varying concentration at a cellular or local tissue level. Thus, it is possible that a T lymphocyte secreting molecules either through an immunological synapse or into the extracellular milieu of an MS lesion, could expose cells to far higher local concentrations.

Due to these problems, I decided to take a candidate approach of investigating LIF as a cytokine, which is already known to have an effect on oligodendroglial cell biology, and try and deliver it locally to OPCs using novel nanoparticle technology.

4.5 Why are Nanoparticles interesting tools in CNS remyelination research?

Much attention has been paid to nanoparticle research in recent years because of their promise over a range of applications, including targeted drug-delivery and possibility to cross the blood brain barrier. In this project, I hypothesised that porous nanoparticles containing a Treg-secreted cytokine, LIF, would, if targeted to OPCs, give a boost to remyelination *in vivo*. Although this strategy is promising, this hypothesis relies on several assumptions: firstly, that these nanoparticles successfully target OPCs in vitro and vivo, and secondly, that these nanoparticles deliver enough LIF to produce an effect. Further investigations need to be done to determine whether nanoparticles will indeed be useful.

With a coating of avidin molecules, biotinylated antibodies can be used to target these nanoparticles - in my case, to NG2 molecules on OPCs, as

from my experiments and the literature, LIF increases proliferation and migration of OPCs in vitro. Experiments to determine whether this strategy effectively targeted nanoparticles to OPCs are currently inconclusive, but earlier work targeting CD4 molecules on T lymphocytes *in vivo* demonstrates that the principle works [182]. However, the nanoparticles in this study were injected intravenously. Further work needs to be carried out to determine the extent of nanoparticle dispersion following injection into the parenchymal tissue of the brain. Previous research into the persistence of nanoparticles *in vivo* has shown that phagocytosis by macrophages is the main limiting factor. This has been demonstrated in the striatum [183]. It will be interesting to see whether microglia (the brain macrophages) are equally limiting. Another variable is that it is difficult to quantify the quantity of LIF contained in each nanoparticle. Attempts have been made to determine this by testing media containing LIF-nanoparticles over 7 days. This showed that around 900pg of LIF was secreted by 1mg of nanoparticles [182] - a very small amount - however, it should still be enough to apply a high local concentration if the nanoparticle is bound to the cell with antibodies. This is the same principle of localised release that the immunological synapse relies on and it may stimulate OPCs sufficiently, if our assumptions are correct.

4.6 Future work

Following on from this, we will be building on our work on LIF-nanoparticles, attempting to discover whether they might be useful for ameliorating demyelination. Once nanoparticle presence in the brain is confirmed, we will inject nanoparticles into demyelinated lesions to determine whether remyelination is improved. If this is the case, the next

step will be to confirm our hypothesis that LIF is targeting OPCs in the environs of the lesion and not other supporting cells, such as microglia. A possible method for this is staining for the proteins involved in the LIF-signalling pathway, such as Stat3. Stat3 is a downstream transcription factor that undergoes dimerisation and subsequent phosphorylation on LIF binding its receptor. Phosphorylated Stat3 can be detected using immunostaining and thus we can determine which cells are activated by LIF signalling. There are reliable markers for the three cell types that would be of most interest in this respect; OPCs (Olig2), microglia (CD68) and astrocytes (GFAP).

Since injecting nanoparticles into the CNS is not a practical delivery method in human MS patients, it will be useful to investigate alternative methods of delivery. Some nanoparticles have been designed to cross the blood-brain barrier, which would be ideal in this situation. These nanoparticles, designed primarily to target white blood cells in the bloodstream, may be too large to cross the blood-brain barrier efficiently, at an average size of approximately 100nm, but this has not been tested. However, if they do not, then it may be possible to encapsulate LIF within nanoparticles that are smaller.

If nanoparticles are shown to be useful delivery tools, then other cytokines could be added, perhaps even in a sequential way to improve remyelination. We know that LIF increases proliferation and migration, which would improve OPC recruitment to MS lesions, but other cytokines such as 9-cis Retinoic acid or BMP inhibitors may be beneficial for promoting OPC maturation if released later in the remyelination process. This could be delivered using a separate nanoparticle strategy, or perhaps using one nanoparticle which has a differential release rate of different cytokines. Therefore nanoparticles may be able to be used as medicine delivery systems containing multiple factors released in a sequential way.

These are experiments of the future, but with the development of polymer chemistry with differential release characteristics, this may be possible.

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